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Antibody biosensors for spoilage yeast detection based on impedance spectroscopy

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ABSTRACT

Brettanomyces is a yeast species responsible for wine and cider spoilage, producing volatile phenols that result in off-odors and loss of fruity sensorial qualities. Current commercial detection methods for these spoilage species are liable to frequent false positives, long culture times and fungal contamination. In this work, an interdigitated (IDE) biosensor was created to detect *Brettanomyces* using immunological reactions and impedance spectroscopy analysis. To promote efficient antibody immobilization on the electrodes' surface and to decrease non-specific adsorption, a Self-Assembled Monolayer (SAM) was developed. An impedance spectroscopy analysis, over four yeast strains, confirmed our device's increased efficacy. Compared to label-free sensors, antibody biosensors showed a higher relative impedance. The results also suggested that these biosensors could be a promising method to monitor some spoilage yeasts, offering an efficient alternative to the laborious and expensive traditional methods.

1. Introduction

Brettanomyces is a frequent spoilage yeast found in alcoholic beverages, especially in wine and cider (Chambers and Pretorius, 2010; Claussen, 1904; Silva et al., 2004). These species are responsible for producing metabolic products that are associated with unpleasant flavors and aromas, mostly caused by volatile phenols. During the aging process, *Brettanomyces* can transform natural constituents of both grape and apple juice, p-couramic and ferrulic acids (hydroxycinamic acids), into 4-ethyl-phenol and 4-ethyl-guaiacol, respectively (Mansfield et al., 2002; Romano et al., 2009; Rosaria et al., 2015; Steensels et al., 2015). Experts describe the resultant unpleasant aroma, known as "Brett character", as barnyard, mousiness, smoky, or horse sweat (Šućur et al., 2016). This effect causes significant economic losses for beverage industries. Moreover, due to their ability to form biofilms, these species can live for extended periods of time and survive conventional sanitation procedures (Agnolucci et al., 2010; Joseph et al., 2007).

Since microorganismal contamination is a substantial threat to this sector over the last few decades, various detection methods were developed. For *Brettanomyces* detection, different direct or indirect techniques exist, such as plating or molecular analysis, respectively (Wedral et al., 2010). However, these methods present many problems for industries. Plating, a commonly used direct detection method, faces long incubation times and risk of contamination. For indirect methods, molecular analyses are expensive and require specialized staff to perform the tests, making these techniques inaccessible to many wineries and ciderhouses. Additionally, both detection methods contain a significant delay between the initial contamination and its subsequent detection, leading to delayed treatment and product loss. Consequently, most of the industries leverage indiscriminate control methods such as, chemical additives (e.g. SO_2, Dimethyl dicarbonate (DMCM), chitosan) (Delfini et al., 2002; Zuehlke et al., 2013), filtration methods (Millet and Lonvaud-Funel, 2000; Morata et al., 2004), physical techniques (e.g. ozone, ultrasonic, electricity) (Puértolas et al., 2009; Santos et al., 2012), etc.

As an alternative to these commercial detection methods, impedance spectroscopy analysis has shown promise for in situ detection of different microorganisms (Yang and Bashir, 2008). By leveraging this technique in combination with a biofunctionalized sensor, it is possible to create a platform for early and specific detection of spoilage yeast species. Moreover, this allows for a real-time analysis of the microorganismal growth by analyzing the resultant changes in the surface's electrical characteristics. In impedance measurements, interdigitated microelectrode (IDE) based sensors are conventionally used, offering a large sensitive area and an accurate response in a limited space (Couniot et al., 2013; Krommenhoek et al., 2006; Ma et al., 2015). For this study, we used biofunctionalized IDE platform to detect *Brettanomyces* growth.

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In the development and the application of new biosensors, biofunctionalization and selection of the bioreceptor element play an important role to correctly immobilize the bioreceptor onto the sensor's surface. A covalent linkage to Self-Assembled Monolayers (SAMs) are usually employed (Baldrich et al., 2008b). SAMs are organized structures of organic molecules that allow the chemical immobilization of different compounds for the selective detection (employing antibodies as bioreceptors). The SAMs are formed and remain strongly attached to the surface of the sensor through their terminal thiol group (Love et al., 2005). Second, to ensure for the selection of the bioreceptor, the antibodies provide a high binding selectivity to proteins of the surface of the cells (Vikholm, 2005).

This work aims to fabricate, characterize and provide experimental evidence of the performance of biosensors for the earlier detection of spoilage yeasts using impedance spectroscopy. For that purpose, a detection platform was created by a biofunctionalizing an interdigitated (IDE) sensor with antibodies (as ligands) linked to Mercaptopropionic acids (MPA). The experiments were performed in a culture media with different strains in stirring conditions to evaluate the specificity and sensitivity of the employed antibody. The biofunctionalized sensor provides a novel method for in-situ detection of spoilage yeast.

2. Material and methods

2.1. Chemical and reagents

The employed chemicals and reagents were the following: Mercaptopropionic acid (MPA) (ref: M5801, Sigma-Aldrich), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (ref: E6383, Sigma-Aldrich), N-Hydroxysuccinimide (NHS) (ref: CAS 6066-82-6, Thermo Scientific), Anti-Brett antibody (Z9b-23A, Lebruns labs), Phosphate buffered saline (PBS) (0.01 M, pH 7.4) (ref: P5368-10PAK, Sigma-Aldrich), ethanol absolute 99.5% (ref: 161086.1211, Panreac), the Malt Extract Broth No. 1 (ref: 02-111-500, Scharlab), the BactoTM Agar (ref: 214010, BD) and ciclohexamide 95% (ref: 357420010, ACROS).

The employed antibody was anti-*Brett*, but the antibody's data sheet reported moderate affinity to a few other non-Saccharomyces yeast known to produce spoilage in wine (some possible strains that will give positive results: *Issatchenkia Orientalis, Zygosocchi ailii, Pichia* Sp., *Candida Glabrata, Issatchenkia Orientalis*, *Torulaspore Pretoriensis*, etc.).

2.2. Biosensor fabrication

The interdigitated (IDE) biosensor was designed and fabricated using microfabrication techniques. A 250 nm-thick gold thin film with a 15 nm chromium adhesion layer was deposited by RF sputtering process onto 3″ oxidized silicon wafers. The overall dimensions of the sensors were 7.5 mm width and 14 mm length. The active sensitive surface was a circle of 6 mm in diameter with microelectrodes of 20 μm width and a separation between them of 30 μm.

Before biofunctionalizing, the sensors were cleaned with acetone and ethanol in an ultrasonic bath for 5 min. The connections were made with wires soldered to each contact path and then isolated with an epoxy resin (ARALDIT Ceys). Finally, the complete system was sterilized at 121 °C for 25 min.

2.3. Biofunctionalization of the sensor

The biofunctionalization was built onto the gold surface of the IDEs of the biosensor. Fig. 1 outlines the functionalization process. The sensors were incubated by immersion with MPA (1 mM) for 2 h and then washed with ethanol to remove the unbounded molecules. To activate the SAM, the sensors were first incubated with EDC (46 mM), and then with NHS (46 mM) for 1 h, respectively. Next, the sensors were rinsed with water to eliminate the excess reagents and avoid unspecific bindings (Zuzuarregui et al., 2015).

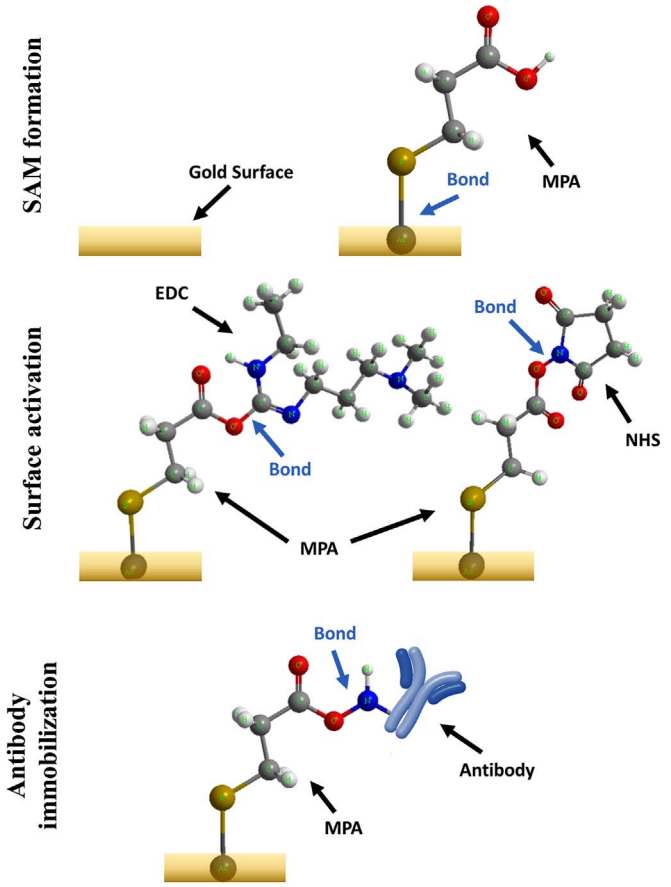

Fig. 1. Self-Assembled Monolayer and antibody immobilization process onto the gold surface of the biosensors.

Finally, after the activation of the SAM, the antibody was immobilized onto the surface of the biosensors. For this study, the device was incubated with 1:1000 antibody concentration overnight and then washed with water to eliminate unbounded antibodies.

2.4. Biosensor characterization

The SAM and antibody immobilization onto the IDE gold surface was characterized using Impedance Spectroscopy (IS) and Fourier Transform Infrared spectroscopy (FT-IR) techniques. IS technique was employed to study the impedance variation before and after antibody immobilization. Measurements were carried out by using a Hioki IM3570 impedance analyzer connected to a custom multiplexer system of 22 independent channels. To evaluate the change of the SAM and antibody electrical behavior during the immobilization, a sweep frequency between 10 Hz and 100 kHz at a constant amplitude of 50 mV was used. FT-IR spectroscopy was also employed to analyze the vibrational modes of the molecular bindings. The samples were measured with the PelkinElmer FT-IR equipment in a range of 3850 cm^{-1} and 650 cm^{-1}. (See Supplementary material for the characterization of the immobilization using the Atomic Force Microscopy (AFM) and Quartz Crystal Microbalance (QCM) techniques)

2.5. Yeasts and culture protocol

Yeast strains from *Brettanomyces bruxellensis*, *Pichia Guillermondi*, *Debaromyces Hansenii* and *Saccharomyces cerevisae* were provided and isolated by Guserbiot S.L. (Vitoria-Gasteiz, Spain). All the strains were stored in a Cryoinstant® mixed vial (ref: 064-TA8276, Scharlab) and preserved at −80 °C. Yeasts were grown in 5 mL of Yeast Media Broth

and maintained inside the incubator at 27 °C for 7–10 days.

The culturing media was designed specifically for spoilage yeast growth (*Brettanomyces bruxellensis*, *Pichia Guillermondi* and *Debaromyces Hansenii*), simulating the conditions in the barrels. It was prepared from dehydrated reagents dissolved in Mili-Q water: Malt Extract Broth No. 1 enriched with cicloheximide (20 mgr/L) and ethanol (6% v/v). For *Saccharomyces cerevisae*, the culture medium was prepared without cicloheximide and ethanol because of the low resistance to those reagents. All the medias were sterilized at 121 °C for 20 min.

Simulating the same storage conditions of wineries, the experiments were carried out in a 1 L reactor (Paredes et al., 2012) placed inside a temperature controlled chamber set to 18 °C, (Barata et al., 2008; Pagliara et al., 2008). Five reactors were employed: one control and four reactors infected separately with *Brettanomyces bruxellensis*, *Pichia Guillermondi*, *Debaromyces Hansenii* and *Saccharomyces cerevisae*, respectively. Stir bars were placed inside the reactors in order to provide a homogenous culture media (100 rpms). In order to compare the sensitivity of the antibodies, ten sensors were introduced in each reactor: five label-free sensors and five antibody biosensors. Label-free and antibody sensors were placed and distributed uniformly inside the bioreactors at a height of 4 cm from the bottom represented in Fig. 2.

Yeast inoculum was prepared setting the concentration at approximately 10^2 CFU/mL using a Neubauer chamber. This value was chosen according to the minimum level of *Brettanomyces* that could cause a repercussion in wine and cider quality. The yeast population must reach a minimum concentration ("critical population") of 10^3 CFU/mL to release a relevant quantity of ethyl-phenols (Hernández and Barbero, 2007; Loureiro and Malfeito-Ferreira, 2006). A Neubauer chamber was employed for a quantitative analysis of the spoilage yeast cells concentration inside the reactors.

2.6. Impedance measurements for growth monitoring

Impedance spectroscopy analysis was employed for yeast strains growth monitoring. This measurements were carried out by using a Solartron 1260 Impedance/Gain-phase Analyzer (Solartron Analytical) connected to a multiplexer system of 24 independent channel (Paredes et al., 2014). Over 8 days, each biosensor´s impedance was measured hourly with a sweep frequency between 10 Hz and 100 kHz at a constant 50 mV amplitude without bias (Tubia et al., 2017).

2.7. Scanning Electron Microscope analysis

At the end of the experiment, Scanning Electron Microscope (SEM) (Phenom ProX) imaging was used to compare the yeast adhesion and the biofilm formation on the surface of the label-free and antibody biosensors at the end of the experiment. To prepare the samples for the SEM imaging, label-free and antibody biosensors were first removed from their respective containers and treated with methanol (Stepanović et al., 2000). Once dry, the biosensors were treated with sputtering for 90 s, covering the surface with palladium.

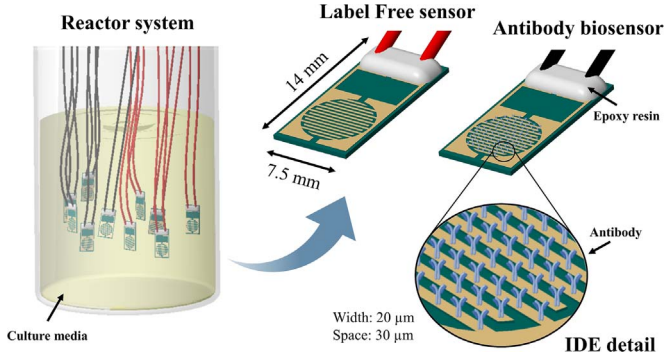

Fig. 2. Setup and different sensors (label-free and antibody) employed during the experiments.

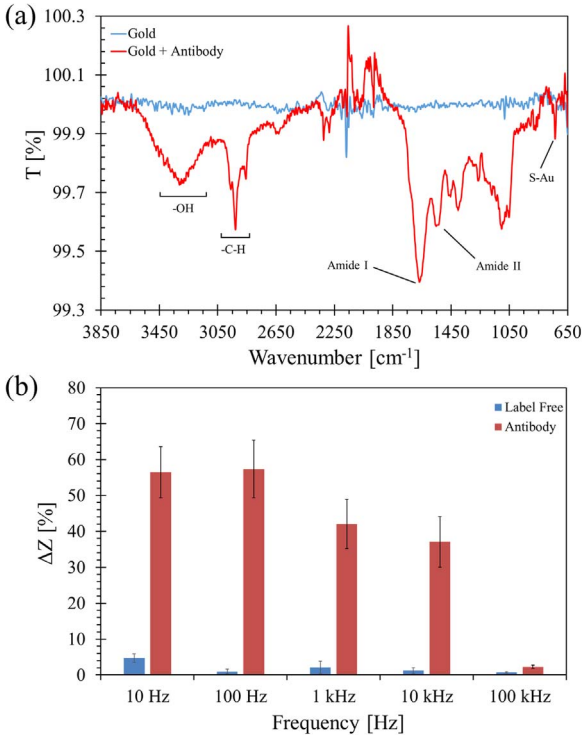

Fig. 3. (a) FT-IR spectrum analysis of the gold and the biofunctionalized gold surface with antibody. (b) Relative impedance variation after the SAM with antibody at different frequencies.

3. Results and discussion

3.1. SAM characterization

3.1.1. FT-IR analysis

In this section, the intermolecular binds were analyzed by infrared spectroscopy. The biofunctionalization of the gold surface with the antibody was confirmed (Fig. 3a) by the presence of peaks in the FT-IR spectrum from 3850 cm^{-1} to 650 cm^{-1} wavenumbers compared to the gold surface (absence of it).

New bands appeared in the spectrum at 1654 and 1534 cm^{-1}, which are characteristics of amide I and amide II bonds (Sasidharan et al., 2013). The peak at 1330 cm^{-1} is characteristic of the O–H bending mode in an intermolecular hydrogen-bonding structure. The peak at 3416 cm^{-1} is due to O–H stretching. 2987 cm^{-1} and 2901 cm^{-1} peaks are assigned to C– H stretching of the alkyl groups of the alkanethiol and the peak at 1407 cm^{-1} to the C–H deformation of the alkyl group. Furthermore, a peak at 1394 cm^{-1} corresponding to O–H bending was observed. A peak at 1067 cm^{-1}, which corresponds to C–OH stretching of the alcohol terminated groups, is also presented. The self-assembled monolayer formation of a covalent gold-sulfur bond was proved by the presence of the large band in the range of 500–750 cm^{-1}, in this case it can be found in 750 cm^{-1}. These peaks were also reported by other authors (Baldrich et al., 2008a; Di Pasqua et al., 2009; Larkin, 2011; Nakamoto, 2009; Tlili et al., 2004). (See Supplementary material Fig. S1 for intermediate steps)

3.1.2. Impedance spectroscopy analysis

Impedance spectroscopy analysis was employed to characterize the surface of the label-free and antibody sensors. Fig. 3b shows the relative impedance variation of label-free sensors and antibody biosensors after the immobilization of the antibodies with a concentration of 1:1000 at

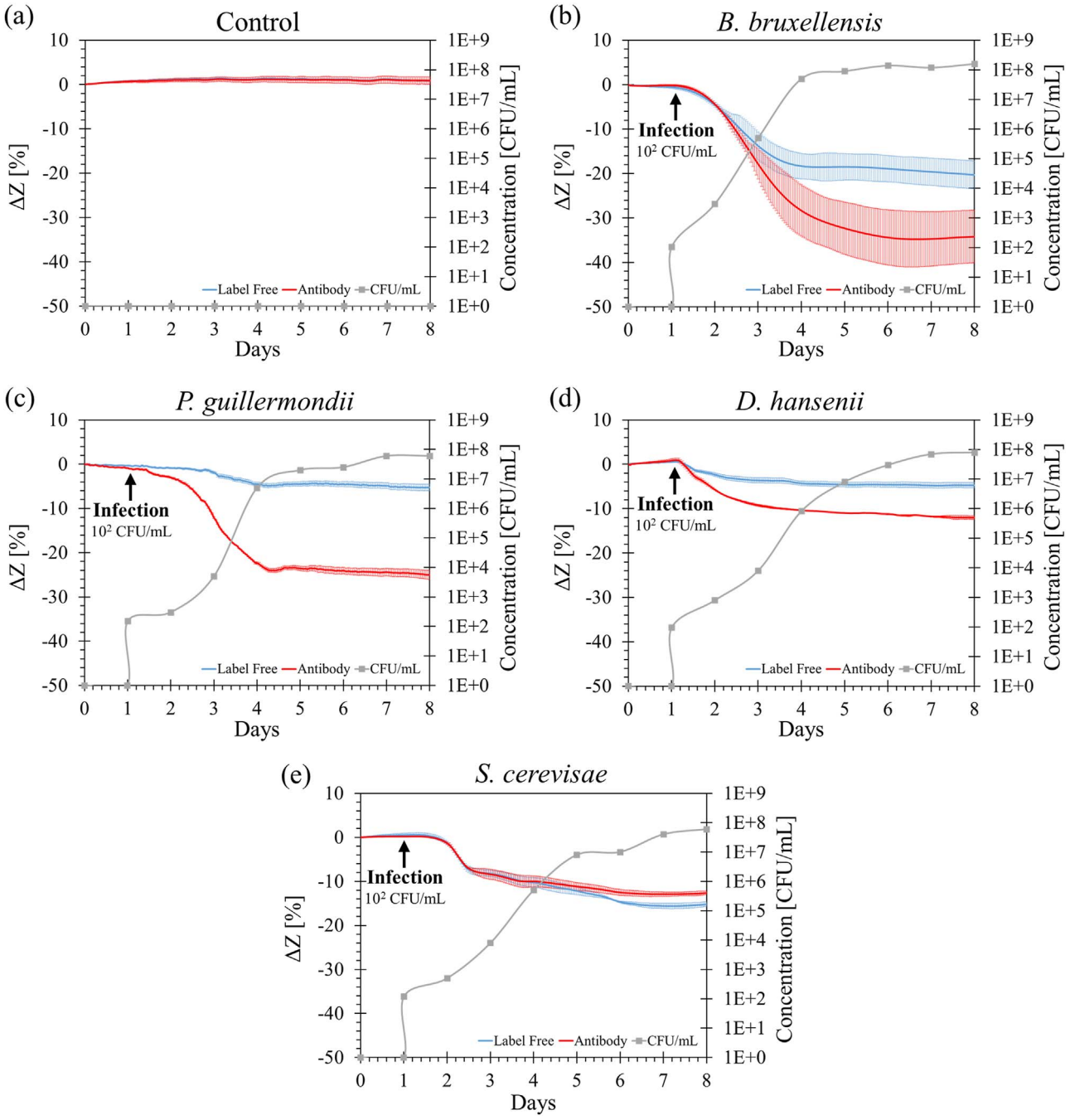

Fig. 4. Relative variation of impedance magnitude for label-free and antibody biosensors at 10 Hz and the concentration of a culture of (a) control, (b) Brettanomyces bruxellensis, (c) Pichia Guillermondi, (d) Debaromyces Hansenii and (e) Saccharomyces cerevisae strains under stirring conditions.

different frequencies in culture media.

Antibody biosensors showed a higher impedance variation after the immobilization of the antibodies compared to label-free sensors. This effect is caused by the insulator behavior of the SAM and the antibodies onto the gold surface (Tlili et al., 2004). In antibody biosensors, low frequencies (10 Hz and 100 Hz) showed more sensitivity (i.e. higher relative impedance change), reaching values of 55% approximately, than higher frequencies (100 kHz). (See Supplementary material Fig. S2 for intermediate steps).

3.2. Growth monitoring of yeast strains

Four yeast strains were analyzed, *Brettanomyces bruxellensis*, *Pichia guillermondi*, *Debaromyces hansenii* and *Saccharomyces cerevisae*. Fig. 4 shows the evolution of the impedance magnitude and cells concentration for the control and the four proposed strains in stirring conditions for 8 days of experiment. The curves illustrate the mean values of the label-free sensors and antibody biosensors including the standard deviation bars over each line for 10 Hz frequency. Of all measured frequencies, low frequency ranges provide better sensitivity for spoilage yeast growth detection. Inoculum of the media was performed after the curves showed a stabilization plateau, in this case, at day 1. Initial concentration in each bioreactor was set at 10^2 CFU/mL of *Brettanomyces bruxellensis*, *Pichia Guillermondi*, *Debaromyces Hansenii* and *Saccharomyces cerevisae*.

Fig. 4a shows the control for label-free and antibody biosensors. During the experiment, the impedance remains constant, suggesting that the antibodies remained attached to the gold surface over this

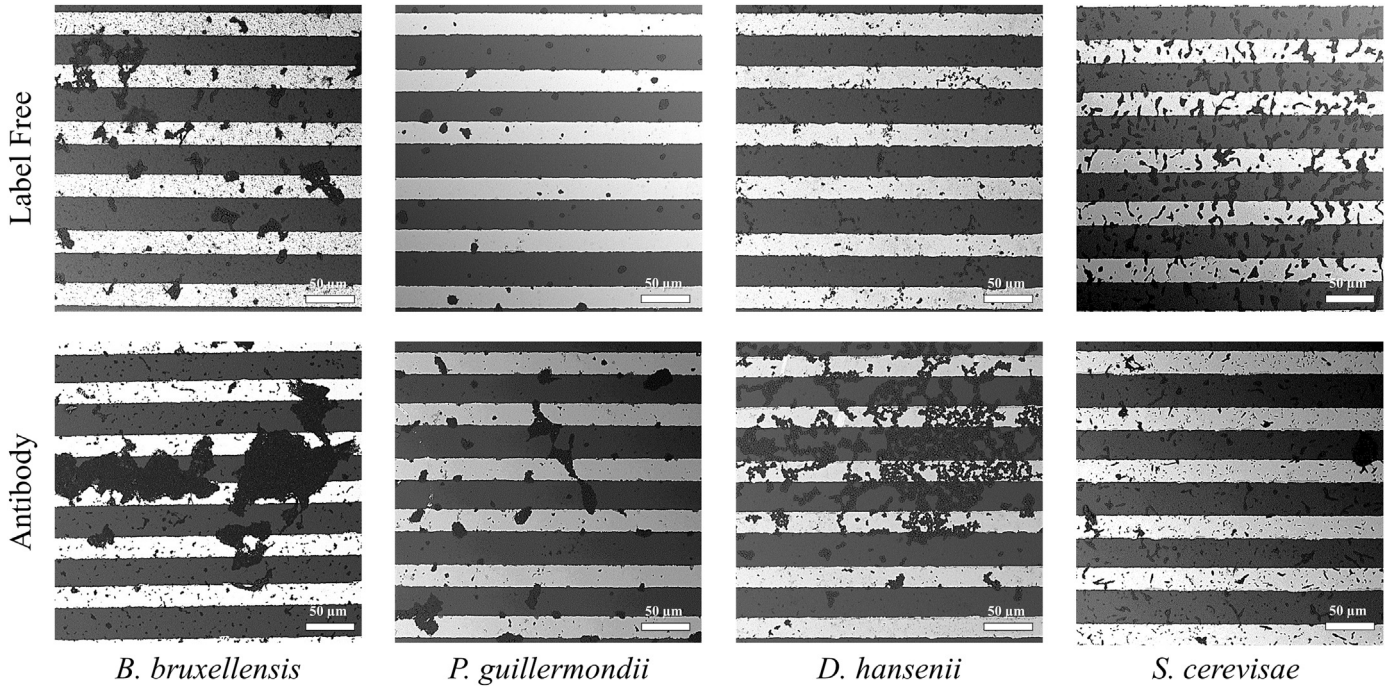

Fig. 5. SEM images of label-free and antibody biosensors with *Brettanomyces bruxellensis*, *Pichia guillermondii*, *Debaromyces hansenii* and *Saccharomyces cerevisae* attached onto the microelectrodes at the end of the experiment.

period. Fig. 4b, c, d and e clearly show changes on impedance one day after the infection with different yeasts. As previously stated, the growth of these cells caused a change on the impedance characteristics. The metabolic activity and the biofilm formation onto the surface of the sensor were responsible of the decrease of the impedance of the culture media for all the yeast strains. The results obtained are corroborated with other studies already published, where a decrease on impedance was related to microbial growth (Radke and Alocilja, 2005; Varshney et al., 2007).

For the impedance measurements of *Brettanomyces bruxellensis* strain (Fig. 4b), the label-free sensors presented variations of 24%, whereas for antibody biosensors, the changes were of 40% (16% increase). This showed that the biosensors with antibody promoted the binding between the cells and the biosensors, generating higher changes on the measured impedance. Besides, the experiments with *Pichia guillermondii* and *Debaromyces hansenii*, also presented a higher impedance changes for biofunctionalized biosensors compared to label-free sensors. For the experiment with *Pichia guillermondii* (Fig. 4c) the impedance changes on label-free sensors were of 6%, whereas for antibody biosensors of 26% (20% more) and for *Debaromyces hansenii* (Fig. 4d), sensors showed 4% and 12% (8% more) of variations in impedance, respectively. These impedance changes corresponded with the increase of the different yeast concentration inside the bioreactor during the experiment as it has been described in Fig. 4.

For the reactors with *Brettanomyces bruxellensis, Pichia Guillermondii* and *Debaromyces hansenii* the curves showed significant variations due to yeast development (metabolic activity and biofilm formation). The stirring of the media allowed nutrient diffusion in a homogeneous manner and oxygenation, facilitating a faster growth and metabolism. Moreover, the stirring condition promoted biofilm formation and cellular attachment due to the shear stress caused by the surface of the sensors (Paredes et al., 2014). In addition, the biosensors with antibodies, showed a higher impedance change due to the higher quantity of cells attached onto the surface.

However, for *Saccharomyces cerevisae* (Fig. 4e), there was little impedance variation between the label-free and antibody biosensors. The label-free sensors and antibody biosensors presented a decrease in impedance of 14% and 12% respectively. However, the label-free sensors presented a 2% higher change on the impedance. It could be caused by a lower binding of the yeasts to the antibody biosensors. Same results were also obtained from two repetitions (See Supplementary material Fig. S6 for more experiment results).

A SEM microscopy was employed to evaluate yeast attachment onto the surface of the sensor. Fig. 5 shows images of label-free and antibody biosensors at the end of the experiment. According to impedance analysis, an increase of yeast cells attached and more developed biofilm structures were observed onto the surface of the biosensor compared to the label-free sensors. In the case of *Saccharomyces cerevisae*, there was less attachment to antibody biosensors. This could be caused because there is no union with the antibodies. While most biofilm development occurred on the gold-plated microelectrode area, shear forces directed some biofilm growth to extend onto the adjacent silicon surface. The silicon surface was also attractive to the yeast (shown in pictures of label-free sensors). Yeast grows all over the surface regardless the material underneath.

It is difficult to match impedance with the SEM images because the impedance measurement is not only affected by the yeasts adhesion onto the surface of the sensor, but also their metabolism. In addition, the impedimetric behavior and growth rate of each yeast strain could be different (for instance the biofilm extracellular matrix density) that affect in a unique way the impedimetric response. Finally, SEM images were selected as a representative area of the whole sensor (0.3% of the total sensitive area).

3.3. Frequency analysis

The effect of the frequency was analyzed in order to identify the frequency that provided higher variations in each case. Fig. 6 presents a comparison of the maximum relative variation of the impedance magnitude at different frequencies (from 10 Hz to 100 kHz) for the control and four yeast strains including the standard deviation bars over each frequency.

The control remained equal for all the frequencies because there was not change on the impedance during the experiment. In the rest of

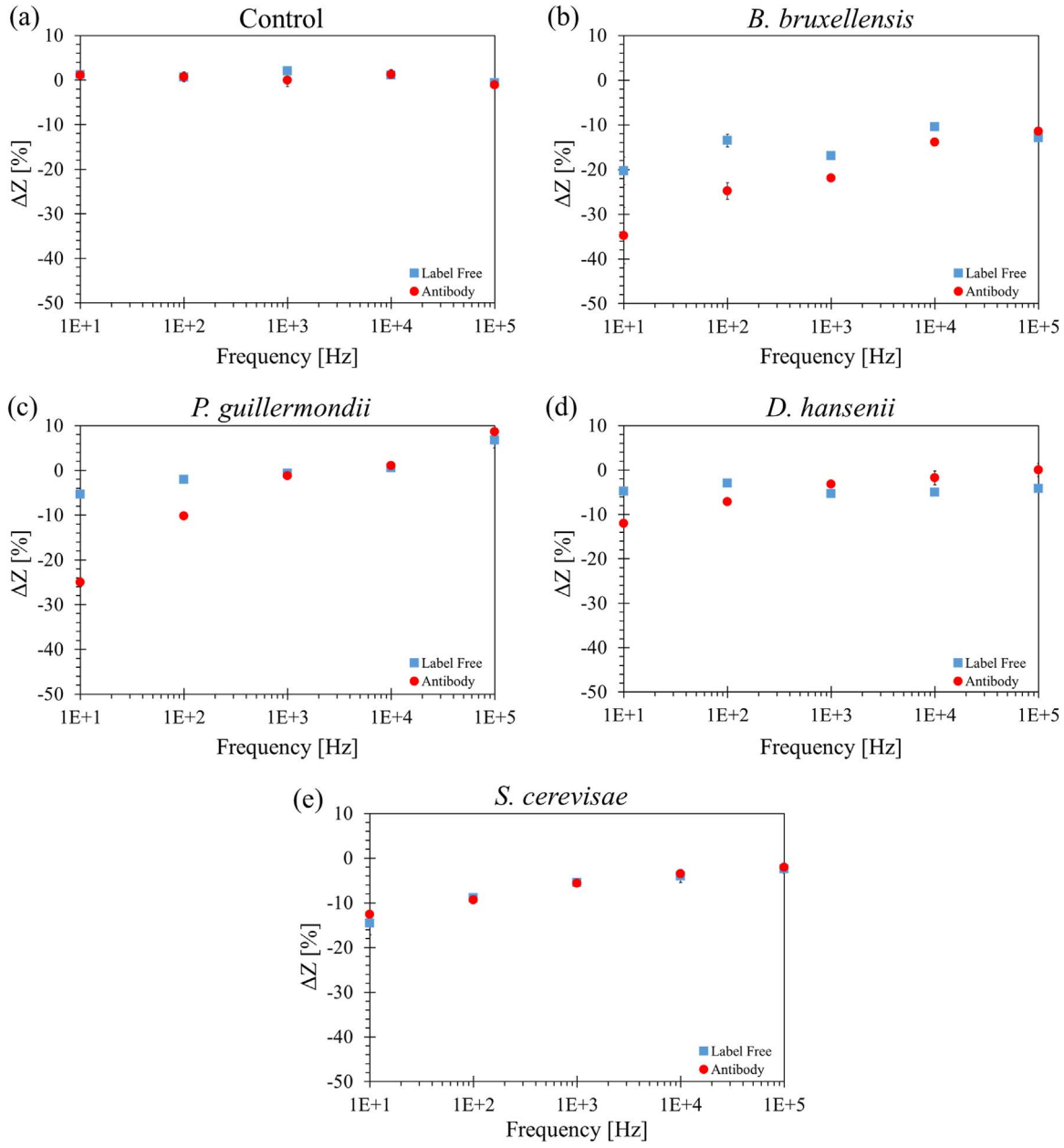

Fig. 6. Relative variation of impedance magnitude at different frequencies (from 10 Hz to 100 kHz) for control media, Brettanomyces bruxellensis, Pichia Guillermondi, Debaromyces Hansenii and Saccharomyces cerevisae strains.

the cases, low frequencies (10–100 Hz) provided higher sensitivity to yeast growth during the experiments.

For low frequencies in *Bretanomyces bruxellensis, Pichia guillermondi* and *Debaromyces hansenii* experiments, the antibody biosensors provide a higher relative variation as a consequence of a higher biofilm coverage over the sensors. Similar results were observed by other authors, where the greatest change in impedance was observed at 10 Hz (Yang et al., 2004). In the case of *Saccharomyces cerevisae*, there is no difference between label-free and antibody biosensors suggesting that there is no specific union between this strain and the antibody.

4. Conclusions

This work presents an antibody detection method of different spoilage yeast species, including *Brettanomyces bruxellensis, Pichia Guillermondi* and *Debaromyces Hansenii*. IDE based sensor were successfully employed to perform impedance spectroscopy analysis under different experimental conditions.

Antibodies were immobilized by a self-assembled monolayer onto the sensor's surface and every step was characterized by FT-IR, QCM, AFM, and impedance techniques ensuring a good bonding.

Experimental results showed that antibody biosensors have higher impedance variations when exposed to their target species, in this case, spoilage yeasts. The specific binding of the antibody promoted a larger and faster biofilm formation onto the surface of the biosensor. Also, it was found that the low-frequency range (10–100 Hz) offered the highest resolution to detect impedance changes for both label-free and antibody sensors.

In conclusion, an in situ and in-real time detection method for spoilage yeast was successfully implemented in the lab. The developed biosensor could be potentially used in wine and cider industries to control unwanted contamination reducing the costs in production lines.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.11.057.

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