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Brettanomyces bruxellensis growth detection using interdigitated microelectrode based sensors by means of impedance analysis

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ABSTRACT

Brettanomyces bruxellensis is considered one of the most relevant spoilage yeasts in the production of alcoholic beverages, especially for wine and cider. During fermentation and later storage, these yeasts can cause changes in the characteristics of the product, ruining the aroma and taste. The presence of *Brettanomyces* causes a decrease in the quality of the final products and important economic losses. The current work presents a detection method based on impedance spectroscopy analysis using label-free interdigitated microelectrode (IDE) based sensors for spoilage yeast detection. Different conditions (static and stirring) were tested in *Brettanomyces* cultures inside reactors in order to evaluate the growth behavior. Our results indicate a faster response and an 8% increase of the relative variation of the impedance under stirring condition due to biofilm formation onto the surface of the sensors. Equivalent circuit analysis also confirmed that the difference was caused by the larger biofilm formation under dynamic conditions. The results suggest that this technology could be applied for the early detection of spoilage yeast in wine and cider industries, providing more efficient methods to achieve a higher quality of the final products.

1. Introduction

Wine and cider fermentation are complex processes that involve the thorough control of many parameters along the different steps of the production. Besides the original quality of the raw material used in the process, the control of both physical and microbiological conditions is critical to achieve the best quality of the final product. Especially during storage or aging, the appearance of spoilage yeast can frequently cause distortions on the organoleptic properties. One of the main problems is a lack of understanding of the mechanisms whereby yeast begin their activity. Therefore, it is still not possible to control the emergence or awaken of spoilage microorganisms inside the barrels.

Among all wine and cider spoilage microorganisms, *Brettanomyces bruxellensis* and *anomalus* are considered the most important cause of the "Brett Character", generating changes of aromas and flavors [1]. Experts describe this aroma as mousiness, barnyard, smoky, or horse sweat [2,3]. This characteristic change is caused mostly by volatile phenols, like 4-ethyl-phenol and 4-ethyl-guaiacol. *Brettanomyces* is capable of transforming hydroxycinnamic acids (*p*-couramic and ferulic acids), natural constituents of both grape and apple juice as well as wine and cider, in those volatile phenols during storage and aging [4–9]. These changes spoil the quality of these fine elaborated beverages. In addition to the decrease of the quality of the product, the appearance of this spoilage yeast conditions could accelerate the deterioration of the inner surfaces of the barrels. Both effects cause significant economic losses for the producers [10]. Moreover, *Brettanomyces* can survive for long periods of time and withstand the sanitation processes regularly applied in wineries, due to its ability to adhere and form biofilms (microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix).

Different direct or indirect detection methods could be used for the detection of Brettanomyces. Within direct methods it is possible to identify two categories: on the one hand, techniques based on microbiological identification such as plating [11]. These are inexpensive and widely used, although present some drawbacks: long incubation times, lab condition, risk in fungal contamination, false positives, etc. On the other hand, techniques based on molecular analysis such as DNA identification are often used because of their good results and their high sensitivity. There are many examples

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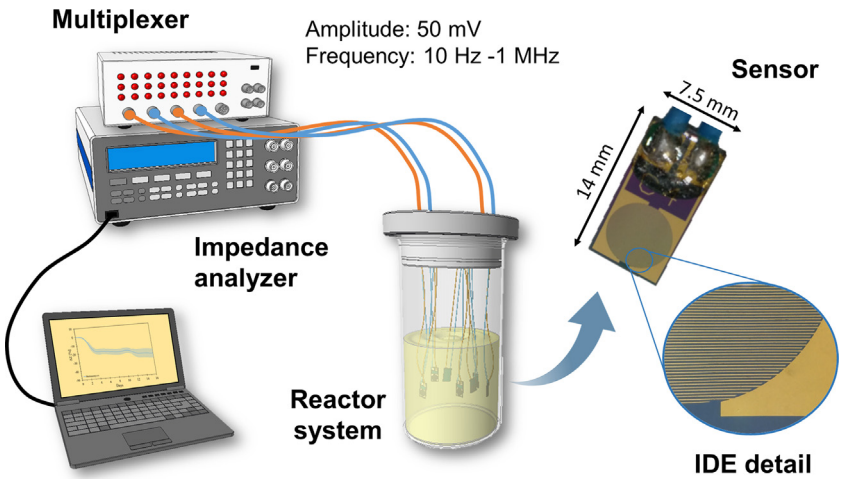

Fig. 1. Experimental setup employed during the experiments composed by an impedance analyzer with a multiplexer connected to label-free sensors.

of these techniques such as quantitative and qualitative PCR using probes for specific sequences [12], hybridization with specific DNA probes and detection with fluorescent microscopy [13], etc. The challenge of DNA methods is the detection of small populations, and even more the identification of alive or dead cells. Furthermore, molecular analysis are expensive techniques that also requires specialized staff to perform the tests, which makes these techniques difficult to be accessed to many wineries.

Indirect detection methods are based on the analysis and quantification of the *Brettanomyces* metabolism products: quantification of ethyl-phenol using gas chromatography and mass spectrometry (GC/MS). However, this kind of analysis requires expensive equipment, and the spoilage yeast to proliferate generating noticeable alterations [14].

As a consequence of such difficulties and the unexpected timing in detection, the majority of the producers move their efforts to implement prophylactic methods in all of their barrels (SO_2 addition is one of the most popular chemicals employed) to avoid the proliferation, instead of treating each barrel individually Zuehlke et al. [36].

In the last decades, impedance spectroscopy based methods have been used as an efficient tool for *in situ* detection of microorganism detection [15]. It is possible that these type of techniques could also be applied for an early detection of spoilage yeast inside barrels improving the production outcome [16]. The detection is based on changes of the electrical characteristics of the medium caused by the presence of microorganisms, either because they grow attached to the surface of the sensor forming biofilms (surface-attached yeasts embedded in a self-produced extracellular polymeric matrix), or because of the metabolic activity changes the chemical composition of the medium. Commonly, for this type of measurements, interdigitated microelectrode (IDE) based sensors are used, offering a large sensitive area and an accurate response in a limited space [17–19]. This method could allow an *in situ* and in real time detection of spoilage yeast growth inside wine or cider barrels and tanks.

In this context, the aim of this work was to provide experimental evidences of the application of this technique in the detection of *Brettanomyces* under different conditions by means of impedance spectroscopy. For that purpose, IDE based sensors were used to monitor the development of spoilage yeasts cultures. The cultures were performed in a media for selective growth of *Brettanomyces* simulating the conditions of the content of the barrels. Moreover, this study presents a comparison of the influence of static and stirring conditions in order to evaluate the adhesion of spoilage yeast onto the surface of the sensor.

2. Materials and methods

2.1. Chemicals and reagents

The employed chemicals and reagents were the following: 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 Bacto™ Agar (ref: 214010, BD), crystal violet (ref: HT90132, Sigma-Aldrich), distilled water (Mili-Q, Millipore) and ciclohexamide 95% (ref: 357420010, ACROS).

The culturing media was prepared for the selective growth of *Brettanomyces* preventing the development of other microbes and simulating the conditions in the barrels [11]. It was prepared from dehydrated reagents dissolved in Mili-Q water: Malt Extract Broth No. 1 enriched with ciclohexamide (20 mgr/L) and ethanol (6% v/v). All the media was sterilized at 121 °C for 20 min.

2.2. Yeasts and culture protocol

Yeast strains from *Brettanomyces bruxellensis* were provided by Guserbiot (Vitoria-Gasteiz, Spain) and were isolated from wine and cider barrels. All the strains were stored in a Cryoinstant® mixed vial (ref: 064-TA8276, Scharlab) and preserved at −80 °C. Yeasts were grown from one frozen bead in 5 mL of Yeast Media Broth and maintained inside the incubator at 27 °C for 7–10 days. Plating method was employed for the isolation and quantification of the colonies.

Microbiological cultures were carried out in a 1 L reactor [20] as it is a common standard to grow biofilms and in order to mimic the real-field conditions in the laboratory. They were placed inside a temperature controlled chamber set to 18 °C, the same aging conditions of wineries [21,6]. Yeast inoculum was prepared setting the concentration at approximately 10^2 CFU/mL. This initial value was chosen according to the minimum level of *Brettanomyces* that could cause an enough metabolism to disturb in wine and cider quality. The yeast population must reach a minimum concentration (called critical population) of 10^3 CFU/mL to begin releasing a measurable quantity of ethyl-phenols. Below this concentration, the yeast is metabolically active, but it is not able to produce detectable levels of spoilage compounds [22].

A quantitative analysis of the spoilage yeasts concentration inside the reactor and also, attached onto the surface of the sensor (number of cells per biosensor) were done. For the yeast concentration inside the reactor, a Neubauer chamber was employed. On the other hand, for the yeast concentration onto the sensor surface,