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Development of an integrated electrochemical system for in vitro yeast viability testing

Andrea Adami *, Cristina Ress, Cristian Collini, Severino Pedrotti, Leandro Lorenzelli

FBK—Fondazione Bruno Kessler, via Sommarive 18, 38123 Trento, Italy

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ABSTRACT

This work describes the development and testing of a microfabricated sensor for rapid cell growth monitoring, especially focused on yeast quality assessment for wine applications. The device consists of a NMOS ISFET sensor with Si_3N_4 gate, able to indirectly monitor extracellular metabolism through pH variation of the medium, and a solid-state reference electrode implemented with PVC membranes doped with lipophilic salts (tetrabutylammonium-tetrabutylborate (TBA-TBB) and Potassium tetrakis(4-chlorphenyl)borate (KTClpB)). The use of a solid state reference electrode enables the implementation of a large number of cell assays in parallel, without the need of external conventional reference electrodes. Microbial growth testing has been performed both in standard culture conditions and on chip at different concentrations of ethanol in order to carry out a commonly used screening of wine yeast strains. Cell growth tests can be performed in few hours, providing a fast, sensitive and low cost analysis with respect to the conventional procedures.

1. Introduction

Throughout history, yeast has proved of undisputed relevance to mankind since it has always been involved in the production of major fermentable products such as wine, beer and bread. During the last two decades, an ever-increasing number of cell biologists and geneticists have widely used yeast in their research labs as it combines the benefits of simple growth requirements with highly conserved eukaryotic cellular pathways. Indeed, similarly to prokaryotes, it is a fast-growing and easy-to-manipulate unicellular organism. On the other hand, being a eukaryotic cell, it is playing a key role as model organism for studying cancer (Simon and Bedalov, 2004) and cell cycle regulation (Stewart et al., 2003). Besides the intensive genome-wide analysis, yeast has been extensively exploited by biotechnologists for the production of vaccines (Garrison and Baker, 1991), enzymes (Elliott et al., 1990), antioxidants, vitamins and flavours (Abbas, 2006).

Moreover, since ethanol is a parameter of considerable economic value for the breweries, wine and biofuel industries, significant research is involved in ethanol tolerance testing of different yeast strains (D'Amore et al., 1990; Graham, 2002). Alcohol toxicity, in fact, inhibits cell growth and adversely affects the fermentation process by stopping further ethanol production (Piper, 1995). Therefore, several studies currently concern wine yeast strains selection in order to check their survival and ability in carrying out difficult fermentation processes with high alcohol concentration (Fujita et al., 2006; Hu et al., 2007).

In the effort to develop and select efficient yeast strains as industrial biocatalysts, high-throughput screenings for large-scale analysis are increasingly required. Conventionally, the best performing microbial strains are firstly evaluated by cell growth monitoring in Petri dishes or by direct microscopic counts. Typically, a number of Petri dishes are prepared with gel culture medium (e.g. based on agar) at different ethanol concentrations. Cells are inoculated and cultures are incubated for 1–3 days. Visual inspection and comparison of dishes provide an evaluation of the ethanol threshold able to stop the yeast replication. Standard methods are therefore time-consuming and labour-intensive, thus limiting parallelization and high throughput selection of the desirable yeast strains. In alternative, other laboratory techniques such as cell counting can be used. This technique measures the cell viability by cell replication rather than cell metabolism and it requires costly equipments such as fluorescence imagers or flow cytometers, which are usually not available in small agro-food companies. In the last three decades, many studies have been focused on the microbial metabolism due to the proven correlation between cell growth and extracellular acidification, oxygen content or ionic conductivity change (Krommenhoek et al., 2008; Huth et al., 1990; Owicky and Parce, 1992; Silley and Forsythe, 1996), enabling the development of microelectronic sensors for culture monitoring, especially based on ion sensitive field effect transistor (ISFET) sensors

* Corresponding author. Tel.: +39 0461 314 497.
E-mail address: andadami@fbk.eu (A. Adami).

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(Yuqing et al., 2003; Lorenzelli et al., 2003) and impedance-based microdevices (Eden and Eden, 1984; Mishima et al., 1991; Asami and Yonezawa, 1995; Gomez et al., 2002; Soley et al., 2005; Krommenhoek et al., 2006; Sherry et al., 2006; Kim et al., 2009). Moreover, multiparametric chips have been proposed to carry out comprehensive studies about physiological state and dynamic behaviour of cell cultures (Krommenhoek et al., 2008; Thedinga et al., 2007). Impedance spectroscopy can measure cell concentration of a culture by using integrated microelectrodes to evaluate the solution impedance in the frequency domain, although it is better suited for adherent cells rather than non-adherent (such yeast), since cell settling can affect the measurement. Oxygen sensors are typically based on optical methods, which are not of practical use for cell cultures because of solution turbidity and presence of bubbles, and Clark's electrodes, which are difficult to be integrated. Then, pH is still the most reliable parameter for cell growth monitoring with integrated sensors. With an approach based on integrated ISFET sensors, it is possible to perform a large number of parallel tests, with obvious benefices for large analysis throughput and yeast selection since it may be unpractical to perform the same analysis on a large number of samples with general purpose pH-metres.

One drawback of many solutions based on potentiometric sensors is the need of bulky external reference electrodes, which limits the actual integration of the system to a limited number of independent measurements or requires the use of salt-bridge for the electrical connection of a single reference with multiple sensors. Many different technological approaches have been presented in the literature for the implementation of integrated reference electrodes. Different possibilities deal with the miniaturisation of standard reference electrode, by integrating electrolyte reservoirs on chip and appropriate electrodes (e.g. Ag/AgCl in KCl). In order to provide the electrical connectivity with inner electrolyte, membranes or microchannels can be implemented on silicon or polymeric devices (Suzuki et al., 1999; Tymecki et al., 2004; Simonis et al., 2004). In a variation of the previous approach, the electrolyte can be implemented as a gel with the appropriate concentration of salts. Coating gel electrolytes with membranes provides a better stability of salt concentration in the electrolyte and therefore of the potential (Zhou et al., 2010). In these configurations, the electrical potential of the electrode is set by the redox equilibrium of species at the electrode surface, as in glass reference electrodes. Therefore, the stability of the potential is directly linked to the chemical stability of the electrolyte and of the electrode. With the small volumes usually implemented with integrated reservoirs, the stability over time is quite limited by the diffusion of ions in the membrane or in the porous plugs or microchannels. The sizes of pores or channels or the membrane properties define the impedance of the electrode (Bosch et al., 2002), reflecting in the electrode sensitivity to noise, leakage currents, etc.

Recently, all-solid-state reference electrodes, based on polymeric layers doped with lipophilic salts to provide electrical conductivity and stable potential, have provided promising results in term of electrical stability and long term viability of the device (Ivanova et al., 2010). In these case, a solid solution of organic salts in a polymeric matrix is coated on electrodes. A solid solution of polymers and lipophilic salts develop a phase boundary potential as function of ion concentration in the membrane $[I^+]$, ion activity in water a_I, and phase transfer energy k_I (Bakker et al., 2004):

$$E_{PB} = \frac{RT}{zF}\ln\left(\frac{k_I a_I}{[I^+]}\right) \quad (1)$$

Most used polymeric membranes for this purpose are based on PVC or on polyurethane blends and have demonstrated good stability of potential to diluted salt solutions (typically <0.1 M) and pH (Ivanova et al., 2010; Mattinen et al., 2009; Lee et al., 1998; Yun et al., 1997; Yoon et al., 2000). In order to provide a fully integrated system, suitability and reliability of such approach need to be tested in real measurement conditions.

This work presents an integrated system based on microsensors for evaluating ethanol resistance of yeasts is presented, which is a test of paramount importance for assessing the quality of yeast strains in wine applications. The presented microsensor is realized with a modified n-MOSFET technology, allowing the realisation of Ion-Sensitive FETs (ISFETs) for the measurement of pH and electrodes made of different materials for the implementation of coated-electrode reference and measurement electrodes. The aim of this paper is to demonstrate the suitability of a fully integrated system for the real application to cellular culture testing, in particular for oenology, by monitoring the acidification of growth medium. In this work, we selected PVC-based membranes for reference implementation because of the availability on the market of a wide choice of chemicals for their implementation, including plasticisers, organic salts and ionophores which might allow the extension of the described activities to ion-sensitive electrode systems (http://www.sigmaaldrich.com). With the described technology, the system can be extended to measurement of several chemical and physical properties of solutions such as conductivity, redox potential and concentration of specific ions.

This work is implemented as follows: in Section 2 "Materials and methods" the solid-state sensors, reference electrode preparation and details of testing procedures are described. In Section 3 "Results and discussion", at first, pH measurements are compared with the trend of cell proliferation measured with cell counters. The detection of the exponential growth phase in the first hours after culture inoculum and the growth rate variation in presence of ethanol is demonstrated by means of pH measurements with both standard instrumentations and pH sensors with external Ag/AgCl reference electrodes. Stability of the electrical potential of polymeric reference electrode is then tested before proceeding with the testing of culture acidification with the fully integrated system at different ethanol concentrations. Results obtained with polymeric reference electrode are also compared with similar measurements performed with integrated ISFETs and conventional reference electrodes. In Section 4 "Conclusions", an overview of results and future work is reported.

2. Materials and methods

2.1. Device design and fabrications

The integrated sensors have been realized with a non-standard fabrication process derived from a 4 μm Al-gate CMOS technology in order to include the realisation of double layer SiO_2/Si_3N_4 gate dielectric for pH sensitivity and electrodes. The choice of gate dielectric material is a key parameter to obtain device performances close to the Nernst's theoretical sensitivity (59 mV/pH at 300 K) and with linear response over the measurement range. Stoichiometric silicon nitride (Si_3N_4), deposited by Low Pressure Chemical Vapour Deposition (LPCVD), allows a good sensitivity and stability with a high compatibility with CMOS technologies and without the need for dedicated equipment. The technology has been simplified by implementing n-channel devices only, since the complementary technology is not required for the realisation of sensors, and n-channel devices have higher carrier mobility. Starting from n-type 4" silicon wafers, ISFETs have been realized on p-well (boron implant and diffusion, final junction depth 4.7 μm) in order to insulate the devices from the n-type substrate. Source and drain n+ regions and substrate contacts have been realized by diffusion from phosphorus pre-deposition with a final junction depth of 750 nm. Ohmic contacts to p-well have been realized with BF_2 implant. Electrical insulation has been provided by a Low Temperature