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Freeze-drying as suitable method to achieve ready-to-use yeast biosensors for androgenic and estrogenic compounds

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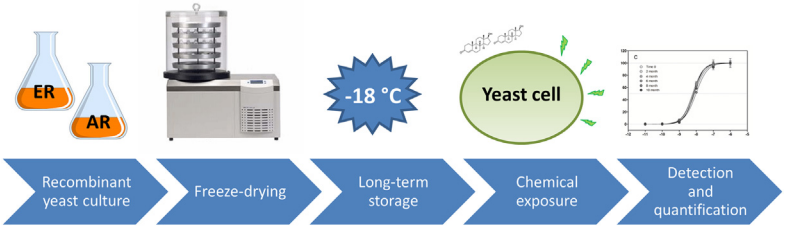
HIGHLIGHTS

- Freeze-drying immobilization to obtain "ready-to-use" versions of yeast biosensors.
- Immobilized yeast cells stored at −18 °C retained viability at least up to 10 months.
- Sensitivity towards androgens and estrogens was comparable to standard assays.
- The new method shortens conventional procedures from 3–4 days to 6 h in non-sterile conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

Recombinant yeast assays (RYAs) have been proved to be a suitable tool for the fast screening of compounds with endocrine disrupting activities. However, ready-to-use versions more accessible to less equipped laboratories and field studies are scarce and far from optimal throughputs. Here, we have applied freeze-drying technology to optimize RYA for the fast assessment of environmental compounds with estrogenic and androgenic potencies. The effects of different cryoprotectants, initial optical density and long-term storage were evaluated. The study included detailed characterization of sensitivity, robustness and reproducibility of the new ready-to-use versions, as well as comparison with the standard assays. Freeze-dried RYAs showed similar dose-responses curves to their homolog standard assays, with Lowest Observed Effect Concentration (LOEC) and Median effective Concentration (EC_{50}) of 1 nM and 7.5 nM for testosterone, and 0.05 nM and 0.5 nM for 17β-estradiol, respectively. Freeze-dried cells stored at 4 °C retained maximum sensitivity up to 2 months, while cells stored at −18 °C showed no decrease in sensitivity throughout the study (10 months). This ready-to-use RYA is easily accessible and may be potentially used for on-site applications.

1. Introduction

Many chemicals and natural products are able to interact with the endocrine system by mimicking or counteracting natural hormones, which may result in the alteration of the correct physiological functioning and, thus, lead to deleterious effects (Duntas, 2014; Patisaul and Adewale, 2009; Waye and Trudeau, 2011). These substances, known as endocrine disrupting compounds (EDCs), are now widespread all over the globe and can exert their action at very low concentrations, representing a real threat for living organisms, including humans (Elsworth et al., 2015; Hu et al., 2009; Jarque et al., 2015; Kidd et al., 2007; Kinch et al., 2015). Effective tools for the fast detection of EDCs are

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consequently needed. The European Regulation No 1907/2006 for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) calls for the development, validation and acceptance of alternative approaches for further replacement, reduction and refinement of animal use in testing of chemicals (OJL396, 2006). Given the high reproducibility and sensitivity, *in vitro* models are pointed out as good alternatives to *in vivo* testing (Brix et al., 2010; Thibeault et al., 2014).

Recombinant yeast assays (RYA) have been proved to be suitable tools for the fast detection and quantification of EDCs either alone or in environmental samples (Brix et al., 2010; Fernandez et al., 2009; Layton et al., 2002; Leskinen et al., 2005). Unlike bacteria, yeast are eukaryotic organisms with folding and post-translational processes similar to vertebrate cells, which result in the correct expression of transfected mammalian receptors. Compared to other more sensitive *in vitro* eukaryotic models, e. g. mammalian or fish cell lines, RYAs are easy to perform, usually less time-consuming, show good sensitivity and high reproducibility, represent relatively low costs and are compatible with immobilization strategies for in-field testing. They are obtained by introducing two foreign elements in a yeast cell, (i) a receptor able to recognize and to bind the ligand of interest, and (ii) a reporter gene whose expression is under control of specific sequences in the gene promoter. Thus, when the ligand binds to the receptor, the new complex receptor-ligand is able to recognize the specific sequences and activates the expression of the gene reporter, which is typically detected by chromogenic (García-Reyero et al., 2001), fluorogenic (Noguerol et al., 2006b) or luminometric methods (Michelini et al., 2008).

The topic of the effective cell immobilization, long-term storage and subsequent fast recovery to achieve ready-to-use versions of yeast-based systems has been widely studied in the past years (Cha et al., 2012; Diniz-Mendes et al., 1999; Lodato et al., 1999). However, while this task has been partially addressed in some industrial processes, e. g. for use in biocatalysts or commercial products, no optimal solutions have been found when applying to biosensors (reviewed in Michelini et al., 2013). Recently, several matrices such as hydrogels (Fine et al., 2006) and polymers (Bittner et al., 2015; Ponamoreva et al., 2015; Roda et al., 2011) were used for cell entrapment with the aim of obtaining ready-to-use versions of standard RYAs to broaden RYAs applicability. Nevertheless, most of these strategies significantly diminished the performance of the assays mainly because of affecting sensitivity compared to regular assays. In addition, relevant parameters to long-term storage, namely stability and durability of cells, usually showed lower performances relatively short time after immobilization.

Freeze-drying is a two-step dehydration process used for the long-term preservation of perishable materials, including living cells. This method has been relatively well characterized and successfully applied in some bacteria-based portable biosensors (Camanzi et al., 2011; Choi and Gu, 2002; Gu et al., 2001; Wenfeng et al., 2013), but almost no information is available for similar approaches in yeast. In this work, we characterized the applicability of freeze-drying methods in yeast biosensors and subsequently optimized existing RYAs to obtain simple and fast ready-to-use versions with high long-term stability and comparable sensitivity to the standard counterparts.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich (St. Louis, USA). Stocks of trehalose and maltose were prepared in concentrations of 40% w/v. Luciferin solution 1 mM was prepared by dissolving D-luciferin sodium salt into 0.1 M citric acid and 0.1 M trisodium citrate dihydrate.

2.2. Strains and plasmids

Saccharomyces cerevisiae strains BMAEREluc/ERα and BMAAREluc/AR were obtained from BMA64-1A (MATa, ura 3-52, trp1Δ2 leu2-3 112his3-11 ade2-1, can1-100, wild type strain W303 (Baudin-Baillieu et al., 1997)). BMAEREluc/ERα (ER-RYA) contains the coding sequence of human estrogen receptor alpha (ERα) cloned into the constitutive expression vector pG-1 and a reporter plasmid carrying a truncated form of *Photinus pyralis* luciferase regulated by the estrogen responsive element (ERE), which serves as a reporter gene (Leskinen et al., 2003). BMAAREluc/AR (AR-RYA) presents similar construction but containing human androgen receptor (hAR) and androgen responsive element (ARE) in the reporter plasmid (Leskinen et al., 2005).

2.3. Recombinant yeast assay (RYA)

Detailed protocol for RYA was described elsewhere (Michelini et al., 2008). Briefly, yeast from frozen stocks stored at −80 °C were reconstituted for three days on agar plates incubated at 30 °C. Transformed clones were grown overnight in complex synthetic dextrose (SD) medium at 30 °C and 160 rpm. Culture OD_{600} was adjusted to 0.4 and grown again to reach OD_{600} of 0.6, the exponential phase. Aliquots of 100 μl were transferred on to a 96-well plate and 1 μl of tested chemical was added in 5 replicates. Testosterone and 17β-estradiol (E2) concentrations ranged from 10^{-11} to 10^{-6} and 1.5×10^{-11} to 3.3×10^{-8} M, respectively, using DMSO (1% v/v) as solvent. DMSO was used as vehicle control. Plates were incubated at 30 °C for 2.5 h. After incubation, 100 μl of luciferin were dispensed in each well and luminescence measured with a Synergy™ multifunctional microplate reader (BioTek, Winooski, Vermont, USA).

2.4. Freeze-drying procedure and RYA with freeze-dried yeast cells

A single colony from an agar plate containing SD medium was grown overnight in liquid SD medium. Two cryoprotectants and several culture densities were tested in the optimization of the freeze-drying process. Culture OD_{600} was subsequently adjusted to 8. Aliquots of yeast culture were mixed with cryoprotectants (trehalose or maltose dissolved in water, 40% w/v) in proportion 1:1 v/v reaching final OD_{600} of 4, and transferred into petri dishes. Petri dishes were shaken to homogenize the mix and frozen at −32 °C for 3 h. Frozen cultures were freeze-dried at 0.120 mbar (−40 °C) for 24 h with Christ™ Gamma 1-16 LSC (Martin Christ, Osterode, Germany). After the freeze-drying process, yeast were reconstituted into complex medium in the volume initially used (culture + cryoprotectant) during 3 h at 30 °C. Aliquots of 100 μL of resuspended yeast were transferred onto each well of a 96-well microplate and subsequently exposed to 1 μl of chemical during 2.5 h at 30 °C. After the incubation, luminescence was measured as in the case of the standard RYA.

2.5. Long-term storage

Yeast cells freeze-dried onto petri dishes were vacuum-sealed (Bag sealer ETA162, ETA a.s., Prague, Czech Republic) and stored at 4 and −18 °C, and long-term stability was assessed by measuring viability and activity at different time points. Yeast stored at 4 °C were tested each month until obtaining no signal (5 months), while yeast stored at −18 °C were tested every two months (2, 4, 6, 8 and 10 months). Results were compared with time point 0 (freeze-dried yeast used immediately after the freeze-drying process). In order to