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O2k-Fluorometry: Amplex® UltraRed using freeze-dried baker's yeast

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1. Introduction

Baker's yeast is an anhydrobiotic organism which is well known for its persistence without water for decades. When rehydrated, it can rapidly restore active metabolism within minutes [1]. In addition, dried baker's yeast has a high level of viability when rehydrated at 30 to 40 °C [1,2]. Commercially available freeze-dried baker's yeast is rehydrated in Na-Phosphate buffer (1 ml). Pipetting up and down 20 times and 10 min on a shaker are necessary to obtain a homogenous cell suspension. Subsequently, another 10 times carefully pipetting up and down are required, immediately prior to adding a subsample of 20 µl into the 2 ml Oroboros O2k chamber (37 °C). Since the cells sediment and clump rapidly, it is necessary to pipette up and down the stock cell suspension again 10 times prior to addition into the next chamber.

2. Chemicals

Respiration medium: Na-PB (50 mM, pH 7.1); or MiR05 or MiR05Cr.

Yeast stock suspension (20 mg/ml):

- Dissolve 20 mg baker's yeast (active dry yeast granula) in 1 ml warm Na-PB.
- Pipette up and down slowly 20 times to get a homogenous cell suspension. Then put the Eppendorf tube on a shaker for 10 min.
- Pipette up and down carefully another 10 times and fill the syringe. Titrate 20 µl of yeast stock suspension into each 2 ml-chamber (the final concentration in the chamber = 0.2 mg/ml; *in the present example 1.5 mg/ml was used*).
- Repeat pipetting up an down 10 times before each addition to the O2k-chamber.

H_2O_2 calibration solution (40 µM stock):

Commercial solution (Sigma-Aldrich 323381 - 25 ml hydrogen peroxide solution 3 wt.%) = 880 mM; prepare fresh:

- H_2O_2 dilution 1 (1:88): add 284 µl of a commercial solution to 20 ml of 10 µM HCl; fill it up to a final volume of 25 ml with 10 µM HCl, to obtain a 10 mM H_2O_2 solution.
- H_2O_2 dilution 2 (1:125): dilute 200 µl of dilution 1 with 10 µM HCl to a volume of 50 ml to obtain the H_2O_2 stock solution of **40 µM.**

Amplex® UltraRed (Invitrogen A 36006): Storage solution=stock solution 10 mM AmR in DMSO. For details see:
» www.bioblast.at/index.php/Amplex_red

Horse radish peroxidase (Sigma-Aldrich P 8250 - 5 kU): Stock solution 500 U HRP/ml MiR05 or MiR05Cr; can be used as storage solution at -20 °C.

CCCP (Sigma-Aldrich C2759): 1 mM stock solution (1.02 mg in 5 ml DMSO). Intact yeast requires much higher concentrations of uncoupler compared to mammalian cells or mitochondrial preparations.

FCCP (Sigma-Aldrich C2920): 1 mM stock solution; now replaced by CCCP
www.bioblast.at/index.php/Bioblast_alert#Bioblast_alert_2013.2802.29:_2013-08-08
The solvent ethanol for FCCP has the disadvantage that it stimulates respiration as a substrate oxidized by yeast. This is avoided by DMSO as a solvent for CCCP.

3. Experimental setup with the Oroboros O2k

Temperature: 37 °C
Gain for OroboPOS: 1
Polarisation voltage for OroboPOS: 800 mV
Gain for amperometric (Amp) sensor: 1000
Polarisation voltage for Amp sensor: 100 mV
Data recording interval: 2 s
Oxygen solubility factor of 50 mM Na-PB: F_M = 0.95 (for MiR05: 0.92)

4. Experimental protocol

Titrations (final conc.)	**Event name**	**Additions [µl]**
Yeast (0.2 mg/ml)*	Yeast	20
Amplex® UltraRed (10 µM)	AmR	2
Horse radish peroxidase (1 U/ml)	HRP	4
SOD (5 U/ml)	SOD	*variable*
H_2O_2 (0.1 µM steps, total 0.2 µM)	H_2O_2	5 + 5 (two steps)
Glucose (20 mM)	Glc (2 M stock)	20
EtOH	EtOH (pure)	40
CCCP (5 µM steps)**	U	10+10+10+10+..
H_2O_2 (0.1 µM steps, total 0.2 µM)	H_2O_2	5 + 5 (two steps)

Reoxygenations were performed with gas injections of pure oxgen.
*Original experiments: 1.5 mg/ml; **FCCP was used, now replaced by CCCP.

5. Results and discussion

In the study presented in Figure 1 (conditions differed from the above recommendations [3]), respiration and H_2O_2 production of yeast (1.5 mg/ml) was studied under hypoxic and hyperoxic conditions, and after stimulation of respiration by glucose. Extracellular H_2O_2-production by intact yeast cells was a pronounced function of environmental oxygen concentration. Oxygen kinetics of respiration (Fig. 1B) resembled closely but not fully that of isolated mitochondria [4], indicating that extracellular oxygen levels provide a good approximation of intracellular conditions, and intracellular oxygen gradients are small. Stimulation of respiration was pronounced with the extracellular substrates glucose and ethanol, and with uncoupler titrations (Fig. 2). In contrast, these additions exerted a minor effect on extracellular H_2O_2 production (Fig. 2).

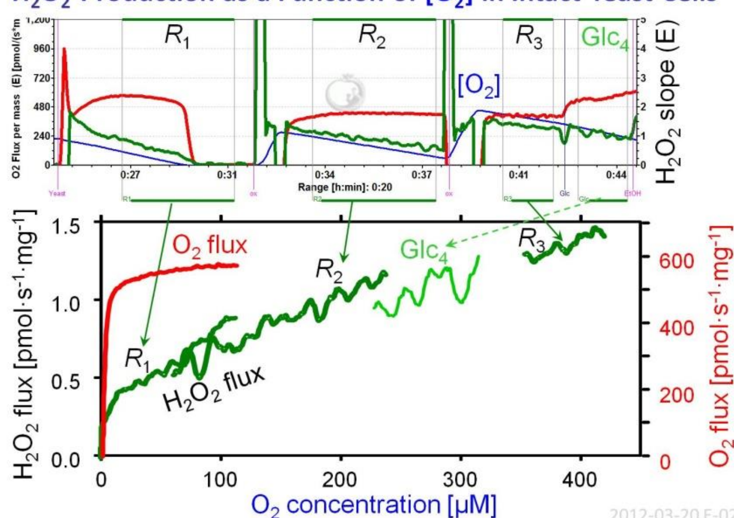

Figure 1. O2k-traces of oxygen concentration (blue plot), oxygen flux (red) and hydrogen peroxide production (green) as a function of time (top) and oxygen concentration (bottom).

Extracellular H_2O_2 production was a linear function of oxygen concentration in the hyperbaric and normoxic range [5]. H_2O_2 production, however, declined steeply in the microoxic range, when the critical oxygen level for respiration was reached (Fig. 1).

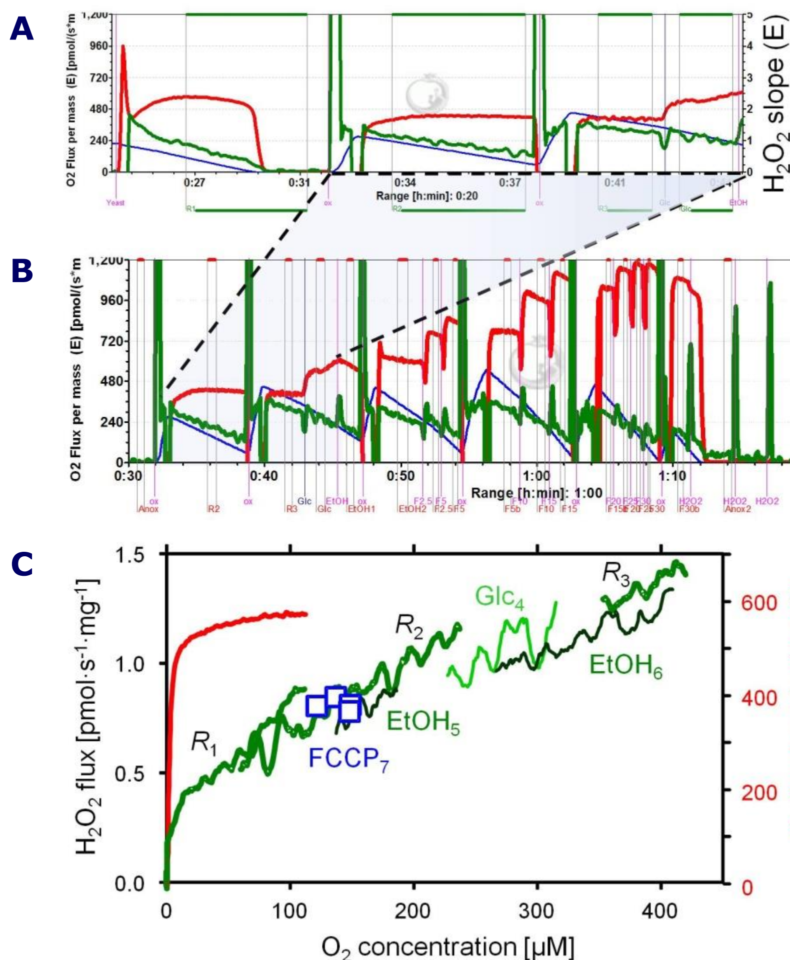

Figure 2. Extension of the experiment shown in Fig. 1 (**A**), by stimulation of respiration by ethanol and uncoupling by FCCP titrations (**B**). Oxygen dependence of extracellular hydrogen peroxide production in different metabolic states of ROUTINE respiration, without external substrates (R_1-R_3), with glucose (Glc_4), ethanol ($EtOH_5$ and $EtOH_6$), and after uncoupling ($FCCP_7$). The subscripts indicate sequential respiratory states (**C**).

6. References

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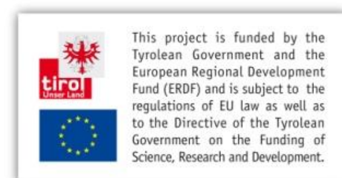

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