OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Protocols

Mitochondrial Physiology Network 18.06: 1-4 (2013)

Version 3: 2013-08-23

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

Mona Fontana-Ayoub[1], Mario Fasching[1], Andrea Eigentler[2], Verena Laner[1], Erich Gnaiger[1,2]

[1]**OROBOROS INSTRUMENTS Corp**
high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
Email: erich.gnaiger@oroboros.at
www.oroboros.at

[2]D. Swarovski Research Laboratory
Department of Visceral, Transplant and Thoracic Surgery
Medical University of Innsbruck, 6020 Innsbruck, Austria
http://wiki.oroboros.at/index.php/K-Regio_MitoCom_Tyrol

Contents

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]. In the present study, commercially available freeze-dried baker's yeast was rehydrated in Na-Phosphate buffer (1 ml). Vortexing for 3 min at 2.200 rpm was necessary to obtain a homogenous cell suspension, immediately prior to adding a subsample of 100 µl into the 2 ml Oxygraph-2k chamber (28 °C). Since the cells sediment and clump rapidly, it was necessary to vortex the stock cell suspension again for 1 min prior to addition into the next chamber.

2. Chemicals

Respiration medium: Na-PB (50 mM, pH 7.1); or MiR05.
Yeast stock suspension (20 mg/ml):

- Dissolve 20 mg baker's yeast (dry powder) in warm 1 ml Na-PB.
- Vortex for 3 min at 2,200 rpm to obtain a homogenous cell suspension.
- Titrate 100 µl of yeast stock suspension into each 2 ml-chamber (final concentration in the chamber = 1.0 mg/ml; in the present example 1.5 mg/ml was used).
- Repeat vortexing for 30 s before each addition to the O2k-chamber.

H_2O_2 calibration solution (56.32 µM stock):
Commercial solution (Sigma-Aldrich 323381 - 25 ml hydrogen peroxide solution 3 wt. %) = 880 mM; prepare fresh:

- Dilution 1 (1:125; solvent H_2O): 200 µl commercial solution in 25 ml (total volume) = 7.04 mM.
- Dilution 2 (1:125; solvent H_2O): 400 µl dilution 1 + 500 µl 1 mM HCl (for stabilization) in 50 ml (total volume) = **56.32 µM stock solution**.

Amplex® UltraRed (Invitrogen A 36006): Storage solution 10 mM; stock solution 1 mM AmR in DMSO. For details see:
>> http://www.bioblast.at/index.php/Amplex_red

Horse raddish 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
http://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 Oxygraph-2k

Temperature: 28 °C
Gain for OroboPOS: 2
Polarisation voltage for OroboPOS: 800 mV
Gain for amperometric (Amp) sensor: 1000
Polarisation voltage for Amp sensor: 100 or 200 mV (=1 or 2 mA light intensity)
Data recording interval: 1 s
Oxygen solubility factor of 50 mM Na-PB: F_M = 0.95
Layout: E1 Fluor_O2_AmR

4. Experimental protocol

Titrations (final conc.)	**Event name**	**Additions [µl]**
Yeast (1.0 mg/ml)*	Yeast	100
Amplex® UltraRed (5 µM)	AmR	10
Horse raddish peroxidase	HRP	4
SOD	SOD	1.7 (not in present exp.)
H_2O_2 (total added 0.5632 µM)	H_2O_2	10 + 10 (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 (additional 0.5632 µM)	H_2O_2	10 + 10 (two steps)

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

5. Results and discussion

Respiration and hydrogen peroxide production of yeast was studied under hypoxic and hyperoxic conditions, and after stimulation of respiration by glucose (Fig. 1). 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 [3], 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).

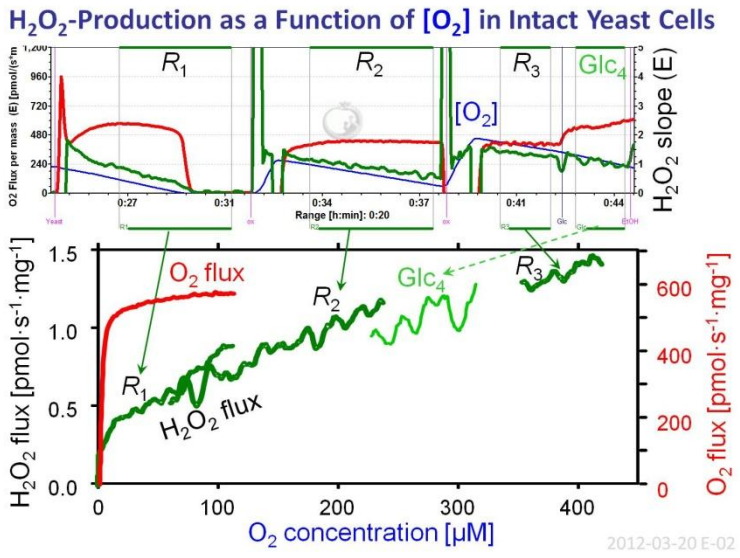

Figure 1. O2k-traces of oxygen concentration (blue plot), oxygen flux (red) and hydrogen peroxide production (green) as a function of time (top) and oxygen concentration (bottom).

Extracellular H_2O_2 production was a linear function of oxygen concentration in the hyperbaric and normoxic range [4]. H_2O_2 production, however, declined steeply in the microoxic range, when the critical oxygen level for respiration was reached (Fig. 1).

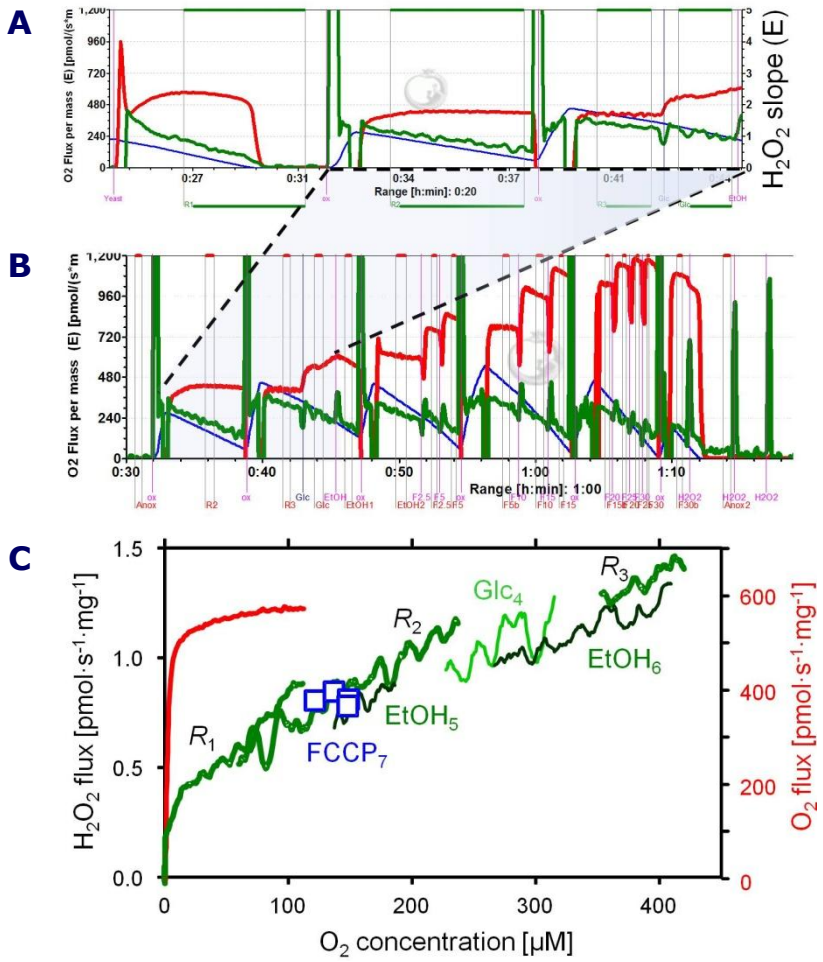

Figure 2. Extension of the experiment shown in Fig. 1 (**A**), by stimulation of respiration by ethanol and uncoupling by FCCP titrations (**B**). Oxygen dependence of extracellular hydrogen peroxide production in different metabolic states of ROUTINE respiration, without external substrates (R_1-R_3),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

6. References

1. Crowe HJ, Carpenter FJ, Crowe M (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60: 73-103.
2. Koga S, Echigo A, Nunomura K (1966) Physical properties of cell water in partially dried *Saccharomyces cerevisiae*. Biophys J 6: 665-674.
3. Scandurra FM, Gnaiger E (2010) Cell respiration under hypoxia: Facts and artefacts in mitochondrial oxygen kinetics. Adv Exp Med Biol 662: 7-25.
4. Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 134: 707-716.

7. Acknowledgements

Contribution to K-Regio project *MitoCom Tyrol*, funded in part by the Tyrolian Government and the European Regional Development Fund (ERDF).
www.oroboros.at/?MitoCom-Tyrol

This project is funded by the Tyrolean Government and the European Regional Development Fund (ERDF) and is subject to the regulations of EU law as well as to the Directive of the Tyrolean Government on the Funding of Science, Research and Development.

Author contributions and publication versions

Prepared by M Fontana-Ayoub, A Eigentler, V Laner and E Gnaiger. Data analysis and final edition by EG.