Antonie van Leeuwenhoek **56**: 211–220, 1989.

Respiratory capacities of mitochondria of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 grown under glucose limitation

HENDRIK VAN URK[1], PETER M. BRUINENBERG[2], MARTEN VEENHUIS[3], W. ALEXANDER SCHEFFERS[1] & JOHANNES P. VAN DIJKEN[1]

[1] *Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands; ([2] Present address: AVEBE, Klaas Nieboerweg 12, 9607 PN Foxhol, The Netherlands); [3] Laboratory of Microbiology, State University of Groningen, Haren, The Netherlands*

Received 10 October 1988; accepted in revised form 10 January 1989

Key words: *Candida,* Crabtree effect, mitochondria, respiration, *Saccharomyces*

Abstract. A comparative study was made of the in vitro respiratory capacity of mitochondria isolated from *Saccharomyces cerevisiae* and *Candida utilis* grown in glucose-limited chemostat cultures. An electron-microscopic analysis of whole cells revealed that the volume density of mitochondria was the same in both yeasts. Mitochondria from both organisms exhibited respiratory control with NADH, pyruvate + malate, 2-oxoglutarate + acetate or malate, and ethanol. The rate of oxidation of these compounds by isolated mitochondria was the same in both yeasts. The rate of oxidation of NADPH by mitochondria from *S. cerevisiae* was 10 times lower than by those from *C. utilis*. However, this low rate probably has no influence on the overall in vivo respiratory capacity of *S. cerevisiae*. The results are discussed in relation to the differences in metabolic behaviour between *S. cerevisiae* and *C. utilis* upon transition of cultures from glucose limitation to glucose excess. It is concluded that the occurrence of alcoholic fermentation in *S. cerevisiae* under these conditions does not result from a bottleneck in the respiratory capacity of the mitochondria.

Introduction

If glucose-limited cultures of *Saccharomyces cerevisiae* and *Candida utilis* are pulsed with excess glucose, differences in their catabolic potential become apparent. Whereas in *S. cerevisiae* a rapid alcoholic fermentation sets in, this phenomenon is almost absent in *C. utilis*. The oxygen consumption rates are about equal under these conditions in both yeasts (Van Urk et al. 1988).

The rapid alcoholic fermentation by *S. cerevisiae* after its transition from glucose limitation to glucose excess has been referred to as the short-term Crabtree effect (Petrik et al. 1983). This phenomenon, which is undesirable

during bakers' yeast production, is attributed to a limited respiratory capacity of *S. cerevisiae* (Käppeli et al. 1985; Rieger et al. 1983). *S. cerevisiae* and *C. utilis*, however, also differ in their anabolic potential. After relief from glucose limitation *S. cerevisiae* does not increase its growth rate immediately, whereas *C. utilis* instantaneously attains a higher biomass production rate (Van Urk et al. 1988).

In view of the differences between the two yeasts in both catabolic and anabolic reactivities and the many interconnections between anabolic and catabolic pathways, it is impossible to draw conclusions with respect to the role of the respiratory capacity in the short-term Crabtree effect from in vivo studies only. It was therefore decided to study the respiratory capacity of the two yeasts via analysis of the respiratory potential of isolated mitochondria, thus avoiding interactions with the cytosolic compartment.

Materials and methods

Organisms and growth conditions

Saccharomyces cerevisiae CBS 8066 and *Candida utilis* CBS 621 were maintained on malt agar slopes. The organisms were grown in a glucose-limited chemostat at $D = 0.2\,h^{-1}$ with ammonium as a nitrogen source as described by Bruinenberg et al. (1983) with the following modifications. The oxygen tension was kept above 20% of air saturation. Glucose concentration in the reservoir medium was 12.5 g/l. In the case of *S. cerevisiae*, the medium also contained 1 ml/l of Tween 80. The pH was kept constant at 5.0 by addition of 2 M KOH and the temperature was controlled at 30°C.

Isolation of mitochondria

Mitochondria from both yeasts were isolated as described by Bruinenberg et al. (1985a) with the following modifications. In the case of *S. cerevisiae*, after harvesting cells (2 g dry weight) were incubated for 30 min at 30°C in 20 ml of a solution containing 5 mM tetra-sodium EDTA and 50 mM dithiothreitol (DTT) in demineralized water. This was necessary for obtaining a sufficient degree of spheroplast formation with Zymolyase. For *C. utilis* this pretreatment was not required (Bruinenberg et al. 1985a). For 2 g of cells (dry weight) 2000 U of Zymolyase (Zymolyase 60,000 or 100,000) in case of *S. cerevisiae* and 250 U in case of *C. utilis* were needed for a satisfactory spheroplast formation.

The cell-free extract obtained (fraction T) was further fractionated as de-

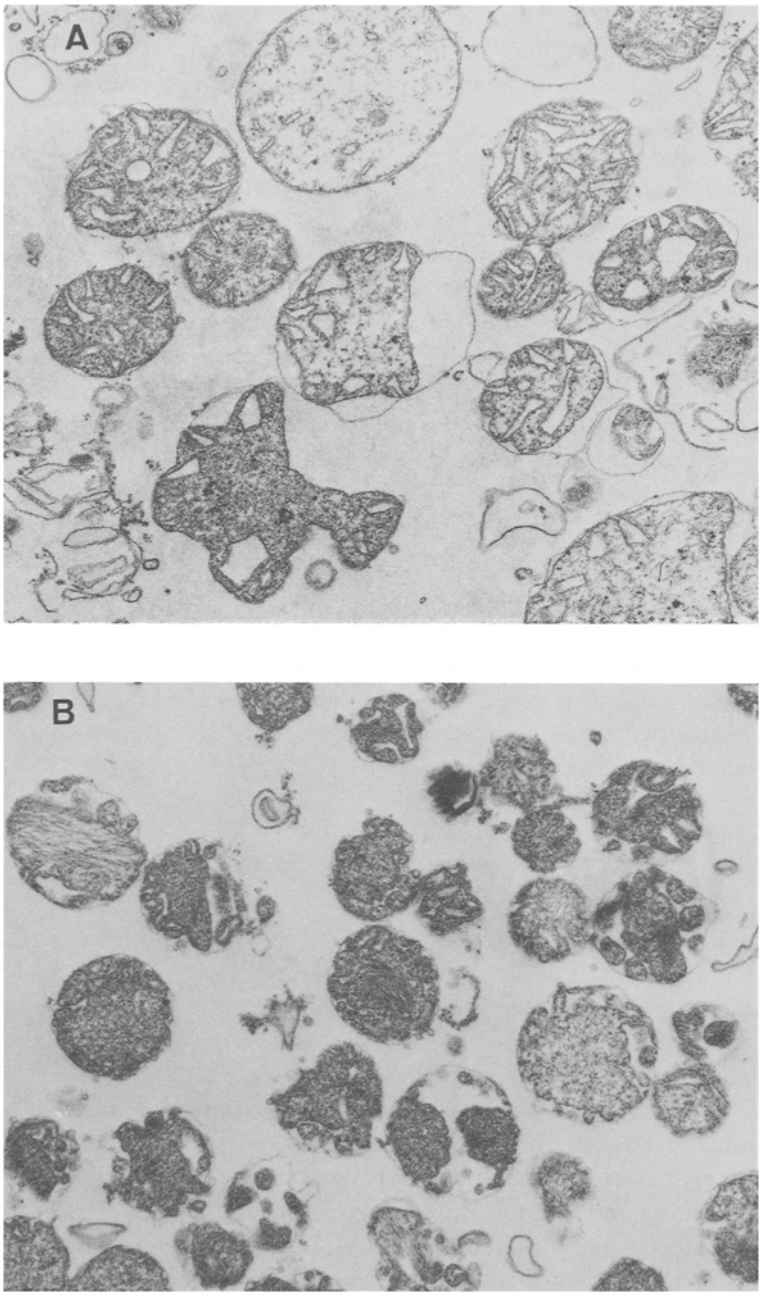

Fig. 1. (A) Electron micrograph of mitochondria isolated from *Candida utilis* (17,000×). (B) Electron micrograph of mitochondria isolated from *Saccharomyces cerevisiae* (11,000×).

scribed by Bruinenberg et al. (1985a). This procedure resulted in a fraction P_1 consisting mainly of mitochondria (Fig. 1), fraction P_2 containing mainly membrane particles, and fraction S containing soluble enzymes.

Polarographic measurements

Oxygen consumption rates were measured polarographically at 30°C according to Bruinenberg et al. (1985a). The substrate concentrations used were: 0.25 mM NADH; 0.75 mM NADPH; 5 mM pyruvate; 2 mM L-malate or 5 mM DL-malate; 5 mM 2-oxoglutarate; 5 mM acetate; or 5 mM ethanol. Respiratory control values (RC) were calculated from the increase in the rate of oxygen consumption upon addition of 0.17 mM ADP. Sensitivity of respiration towards rotenone was tested at a concentration of 8 μM.

Enzyme assays

NADH dehydrogenase (EC 1.6.99.3) activities were assayed as described by Bruinenberg et al. (1985a). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed as described by Bruinenberg et al. (1983). Protein was determined with bovine serum albumin (Sigma, fatty acid-free) as a standard according to the Lowry method.

Electron microscopy

Electron micrographs of mitochondrial fractions were obtained as described by Bruinenberg et al. (1985a).

Measurements of the mitochondrial contents of cells

Measurements were performed via electron micrographs of whole cells. The relevant parameters were determined by screening cell areas (without the cell wall) and mitochondrial areas with a computerized detector. By measuring these two-dimensional parameters on about 200 cell sections taken at random, the ratio of the three-dimensional volumes of mitochondria and cells was determined. This method has been evaluated by Weibel and Bolender (1973).

Biochemicals

Zymolyase preparations were obtained from the Kirin Brewery, Japan. L-malate was from Sigma. Other biochemicals were from Boehringer.

Since commercial preparations of NADH contain ethanol, it was necessary to remove this compound for the study of mitochondrial NADH oxidation. This was accomplished as follows: 100 mg NADH was added to a cell suspension of *C. utilis* (125 mg dry weight in 2 ml of distilled water). This suspension was incubated at 30°C and was sparged with air. After 30 min cells were removed by centrifugation. This procedure resulted in a 30-fold reduction of the ethanol content of the NADH preparation which then was suitable for application in the study of mitochondrial respiration. The recovery of NADH after the procedure was about 80%. The ethanol content of the NADH preparation was determined polarographically using 8 U/ml alcohol oxidase (EC 1.1.3.13). The amount of oxygen consumed was linearly proportional to the ethanol concentration in the assay. The alcohol oxidase used in this assay was purified as described by Verduyn et al. (1984). The preparation was free of NADH oxidase activity.

Results and discussion

Mitochondrial content of cells from chemostat cultures

Cultures of *Candida utilis* and *Saccharomyces cerevisiae* exhibit no important difference in their respiratory activity during transition from glucose-limited conditions to glucose excess (Van Urk et al. 1988). In order to further substantiate evidence that both yeasts possess equal respiratory capacities when cultivated under glucose-limited conditions, the volume fraction of the mitochondria in the cells was determined. The quantitative analysis of electron micrographs of cell sections revealed that *S. cerevisiae* and *C. utilis* grown under the described circumstances contained, respectively, 9 ± 1 and 12 ± 3 vol. % of mitochondria. It can, therefore, be concluded that both organisms contain equal volumes of mitochondria under these conditions.

Isolation of mitochondria from chemostat cultures

For the isolation of mitochondria the procedure of Bruinenberg et al. (1985) was adopted. A few modifications were required for successful isolation of mitochondria from *S. cerevisiae*. A pretreatment of cells with EDTA and DTT was required in order to obtain a satisfactory rate of spheroplast formation

with Zymolyase. This was not required in the case of *C. utilis*, as has also been observed by Bruinenberg et al. (1985a). Spheroplasts of *S. cerevisiae* thus obtained were very fragile and unstable. Even in solutions containing 2 M sorbitol premature bursting of spheroplasts, as measured by the release of glucose-6-phosphate dehydrogenase, was observed. This problem could be circumvented by adding Tween 80 to the growth medium which greatly improved the stability of spheroplasts.

When cell-free homogenates were subjected to differential centrifugation, the recovery of protein in the mitochondrial fraction was higher for *S. cerevisiae* (Table 1). However, because the fractions were not highly purified, this difference in protein recoveries is not sufficient for a higher mitochondrial content in *S. cerevisiae* and cannot be considered as contradictory with the above conclusion from electron-microscopic observations.

Electron microscopy of the mitochondrial fractions revealed that they mainly consisted of mitochondria with intact outer membranes (Fig. 1). Also other criteria for intactness of the mitochondria such as respiratory control (Table 2) and a high recovery of NADH dehydrogenase (Table 1) were met. In the case of *S. cerevisiae*, however, only approximately 50% of the NADH dehydrogenase was recovered in the mitochondrial fractions P_1 and P_2 (Table 1). Apparently in *S. cerevisiae* this enzyme easily dissociates from the membrane during fractionation.

Oxidation of NADH by mitochondria

In order to establish the capacity of the electron transport chain, the rate of

Table 1. Distribution of NADH dehydrogenase (NADH-DH) and protein (mg) over the various fractions. The fractions P_1 (mitochondria), P_2 (membrane particles) and S (soluble enzymes) were obtained from fraction T (total) as described in the methods section. Recoveries are given as % of the total (in fraction T), specific activities of NADH-DH are presented as $\mu mol \times min^{-1} \times$ (mg protein)$^{-1}$.

	S. cerevisiae				*C. utilis*			
Fraction	Protein	Rec.	NADH-DH	Rec.	Protein	Rec.	NADH-DH	Rec.
T	74	–	1.84	–	44	–	4.04	–
P_1	19	26	2.57	34	8	18	12.50	61
P_2	10	14	1.76	10	5	12	1.83	5
S	36	48	1.32	40	29	65	1.25	18
Total recovery		88		84		95		84

NADH oxidation by mitochondria was determined. Exogenous NADH was oxidized at approximately the same rate by the mitochondria of *S. cerevisiae* and of *C. utilis* (Table 2). In both yeasts, oxidation of exogenous NADH was insensitive to rotenone. The finding that mitochondria of both yeasts oxidized exogenous NADH at approximately the same rate does not necessarily imply that the oxidative capacity of the electron transport chain for NADH is the same in these mitochondria. This is due to the fact that in yeasts two NADH dehydrogenases are present (Von Jagow & Klingenberg 1970). One is located on the outer side of the inner mitochondrial membrane, which is functioning in the oxidation of NADH generated in the cytoplasm. The other NADH dehydrogenase is located at the inner surface of the inner membrane and is functioning in the oxidation of NADH, generated in the mitochondrial matrix by the enzymes of the citric acid cycle. Since the inner mitochondrial membrane is impermeable to reduced pyridine nucleotides (Von Jagow & Klingenberg 1970), estimation of the capacity of mitochondria for the oxidation of exogenous NADH does not reflect the overall NADH oxidation capacity.

For the estimation of their overall NADH oxidation capacity, mitochondria were sonicated for various periods of time in order to unmask the latent internal NADH oxidation capacity. The results in Fig. 2 show that upon sonication additional NADH oxidation activity becomes apparent. It is clear

Table 2. Oxidation of substrates and respiratory control values (RC) by the mitochondrial fractions (P_1) isolated from *S. cerevisiae* and *C. utilis*. Activities were measured in the presence of ADP and are expressed as μmol $O_2 \times min^{-1} \times$ (mg protein)$^{-1}$.

Substrate	*S. cerevisiae*		*C. utilis*	
	Activity[1]	RC	Activity[1]	RC
NADH[2]	0.36	2.5	0.22	1.7
NADPH	0.02	1	0.20	2.0
Pyruvate+malate	0.19	1.7	0.16	2.6
2-Oxoglutarate+malate	0.21	3.4	0.20	2.9
2-Oxoglutarate+acetate	0.29	2.2	0.16	3.7
Pyruvate	0.03	1	0.02	1
Malate	0.03	1	0.03	1
2-Oxoglutarate[3]	0.02	–	0.03	–
Acetate[3]	0	–	0	–
Ethanol	0.29	2.2	0.28	2.3
Acetaldehyde	0.43	2.2	–	–

[1] Variation in activities was about 25%.

[2] This activity was measured after removing ethanol from the Boehringer preparation. Ethanol was removed as mentioned in "Materials and methods".

[3] Measured without ADP. 0 Not detectable. – Not measured.

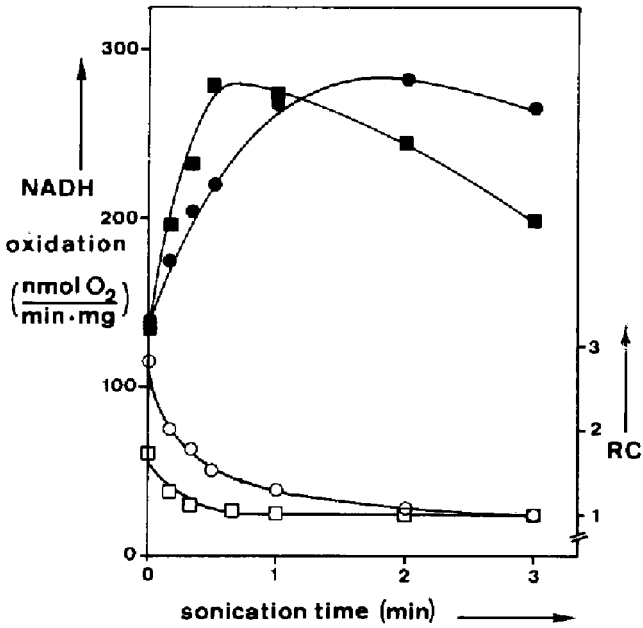

Fig. 2. Release of NADH oxidation capacity (assayed in the absence of ADP) upon sonication of mitochondria isolated from *S. cerevisiae* and *C. utilis* grown at $D = 0.2\,h^{-1}$. Open symbols: RC values; closed symbols: NADH oxidation rates. Circles: *S. cerevisiae*; squares: *C. utilis*.

that both yeasts contained the same amount of latent NADH oxidation capacity. In *C. utilis* this activity was more susceptible to prolonged sonication than in *S. cerevisiae*. As reported previously (Bruinenberg et al. 1985b) this treatment also results in a loss of respiratory control (Fig. 2). The extra NADH oxidase which became detectable upon sonication differed in rotenone sensitivity: In *S. cerevisiae* this NADH oxidase, like the external NADH oxidation activity, was insensitive to rotenone. In mitochondria of *C. utilis*, however, the inner NADH oxidase was nearly completely inhibited by rotenone.

Although the rate of NADH-dependent oxygen consumption by mitochondria of the two organisms did not differ significantly (Fig. 2) the NADH dehydrogenase activities in the mitochondrial P_1 fractions from *C. utilis* were five-fold higher than in those of *S. cerevisiae* (Table 1). Apparently the NADH dehydrogenase assay is not a good measure for the overall capacity of the respiratory chain.

Oxidation of NADPH by mitochondria

Sonication of mitochondria did not result in a release of extra NADPH

oxidation activity, as compared to the activity of mitochondria before sonication (not shown). This was in accordance with the results obtained by Bruinenberg et al. (1985b). A significant difference between the two types of mitochondria with respect to the rate of oxidation of NADPH is shown in Table 2. Mitochondria from *S. cerevisiae* had an NADPH oxidation activity which was 10 times lower than that of mitochondria from *C. utilis*. The physiological background of the different activities found may be related to the high, $NADP^+$-dependent, glucose-6-phosphate dehydrogenase activity in *C. utilis* (namely 1.1 U.mg of soluble protein^{-1}) which is 10 times higher than the activity found in *S. cerevisiae* (namely 0.13 U.mg of soluble protein^{-1}). Therefore, *C. utilis* may need this NADPH oxidation capacity to reoxidize NADPH that is overproduced, which may not be the case in *S. cerevisiae*. Comparative studies on other Crabtree-positive and Crabtree-negative yeasts may further elucidate the physiological significance of the high NADPH oxidation capacity in mitochondria from *C. utilis*. From meassurements discussed below, it may be concluded that the restricted NADPH oxidation capacity of mitochondria from *S. cerevisiae* probably will have no consequences for the in vivo respiratory capacity of this yeast.

Oxidation of other substrates by isolated mitochondria

Besides NADH and NADPH, various other compounds were tested as substrates for the in vitro oxidation by isolated mitchondria. The rates of oxidation of ethanol, of pyruvate in the presence of malate, and of 2-oxoglutarate in the presence of malate or acetate, were similar in the mitochondria of the two yeasts. With these substrates, respiratory control was observed.

Acetaldehyde oxidation was tested with mitochondria isolated from *S. cerevisiae*. Acetaldehyde can be oxidized using either NAD^+ or $NADP^+$ as a cofactor. In view of the low rate of NADPH oxidation by mitochondria from *S. cerevisiae*, the possibility should be considered that in this yeast regeneration of $NADP^+$ might be a bottleneck in the oxidation of acetaldehyde. However, the results presented in Table 2 show that acetaldehyde is oxidized at a rate, similar to that of the other substrates used. Moreover, the oxidation rates of the TCA cycle intermediates tested are similar in both types of mitochondria. These results indicate that $NADP^+$-dependent steps probably play no role of importance in the oxidation of intermediates.

The above results support the hypothesis (Van Urk et al. 1988) that the rapid alcoholic fermentation in *S. cerevisiae* does not result from a limited capacity of the mitoochondrial respiratory system. Both the in vivo and in vitro respiratory capacities in *S. cerevisiae* are similar to those of *C. utilis*, a yeast which does not perform alcoholic fermentation upon transition to glucose excess.

Acknowledgements

We thank Professor J.G. Kuenen for valuable discussions and Mrs. W.H. Batenburg-van der Vegte for the preparation of the electron micrographs.

References

Bruinenberg PM, Van Dijken JP & Scheffers WA (1983) An enzymic analysis of NADPH production and consumption in *Candida utilis*. J. Gen. Microbiol. 129: 965–971

Bruinenberg PM, Van Dijken JP, Kuenen JG & Scheffers WA (1985a) Critical parameters in the isolation of mitochondria from *Candida utilis* grown in continuous culture. J. Gen. Microbiol. 131: 1035–1042

Bruinenberg PM, Van Dijken JP, Kuenen JG & Scheffers WA (1985b) Oxidation of NADH and NADPH by mitochondria from the yeast *Candida utilis*. J. Gen. Microbiol. 131: 1043–1051

Käppeli O, Arreguin M & Rieger M (1985) The respirative breakdown of glucose by *Saccharomyces cerevisiae*: An assessment of a physiological state. J. Gen. Microbiol. 131: 1411–1416

Petrik M, Käppeli O & Fiechter A (1983) An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum:* Involvement of short- and long-term regulation. J. Gen. Microbiol. 129: 43–49

Rieger M, Käppeli O & Fiechter A (1983) The role of a limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae*. J. Gen. Microbiol. 129: 653–661

Van Urk H, Mak PR, Scheffers WA & Van Dijken JP (1988) Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. Yeast 4: 283–291

Verduyn C, Van Dijken JP & Scheffers WA (1984) Colorimetric alcohol assays with alcohol oxidase. J. Microbiol. Methods. 2: 15–25

Von Jagow G & Klingenberg M (1970) Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. Eur. J. Biochem. 12: 583–592

Weibel ER & Bolender RP (1973) Stereological techniques for electron microscopic morphometry. In: Hayat MA (Ed) Principles and Techniques of Electron Microscopy, vol 3. New York/Cincinatti/Toronto/London/Melbourne: Van Nostrand Reinhold Company, pp. 237–296