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Thermotolerant *Candida blankii* Yeast Strain: Biochemical Properties with a Special Reference to Bioenergetics

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Summary

Candida blankii cells readily utilized both fermentable and nonfermentable substrates at 30–45 °C, with the upper temperature limits being at 43–45 °C. No considerable growth was observed at 20–24 °C. The respiratory pattern in cells grown on glucose, succinate or soluble paraffins was almost the same: respiratory rates reached their maxima at the exponential growth phase; exponentially growing cells displayed only the cytochrome oxidative pathway; in cells harvested at the stationary growth phase, both the cytochrome and alternative pathways coexisted. When C. blankii cells were grown on soluble paraffins, succinate or low (derepressible) concentrations of glucose, the oxidative phosphorylation system predominated in the energy budget of the cell. Moreover, the higher temperature of growth (in the range of 30–43 °C) – the greater engagement of the oxidative phosphorylation system occurred. Mitochondria isolated from cells grown at 43 °C on sucrose or succinate possessed the respiratory chain with all three points of energy conservation.

Thus, some important regularities providing better understanding of mechanissm underlying growth of lower eukariotes at high permissible temperatures were revealed. It is becoming evident that 43–45 °C is still the temperature regime at which the respiratory chain functioning is not restricted possibly by balanced changes in the barrier properties of the inner mitochondrial membrane.

Keywords: yeast, thermotolerant, thermophilic, energy metabolism, mitochondria

Introduction

There is currently a wide interest in physiology and biochemistry of microorganisms, growing at elevated and, particularly, extreme temperatures. However, the bulk of the studies available has dealt with thermophilic bacteria *(1–7)*.

Information concerning thermotolerant and thermophilic yeast species is not so abundant. The interest of researchers was largely focused on taxonomy and physiology of thermotolerant yeast species found in complex biocenoses *(8,9)* or producing lipids *(10)* or thermostable enzymes *(11,12)*. Energy metabolism of thermotolerant yeast species was only fragmentarily documented, if not ignored.

The goal of this paper is to describe biochemical properties, with special reference to bioenergetics, of the newly obtained thermotolerant *Candida blankii* yeast species with unusually high (43 °C) upper temperature growth limit.

Materials and Methods

The thermotolerant yeast isolate was obtained from hot wastes of oil-refinery plant (Ufa, Russia). The isolate was purified and identified as *Candida blankii* by its growth requirements. Cultures of *C. blankii* were maintained at 4 °C on YPD-agar slopes and grown with shaking at different temperatures (ranging from 24 to 45 °C) in medium containing (g/L): $MgSO_4 \cdot 7H_2O$ 0.7; KH_2PO_4 0.5; $K_2HPO_4 \cdot 3H_2O$ 0.1; NaCl 0.5; $(NH_4)_2SO_4$ 0.6; $CaCl_2$ 0.1; yeast extract (Difco, USA) 2.0 and (mg/L): KJ 0.1, $CuSO_4 \cdot 5H_2O$ 0.04, $MnSO_4$ 0.4, $FeCl_2 \cdot 6H_2O$ 0.2, $Na_2MoO_4 \cdot 2H_2O$ 0.2, $ZnSO_4 \cdot 7H_2O$ 0.4, H_3BO_3 0.5; biotin 0.002, supplemented with 5% glucose, 1.5% sucrose, 1.5% succinate or 2% octadecane (soluble paraffin) as the sole carbon and energy sources. The pH was adjusted to 5.2 by the addition of KOH. Growth was monitored by measuring the increase in adsorbance at 530 nm or by determining dry weight. At specified time points, cells were collected by centrifugation at 3200 g for 10 min,

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washed twice by resuspension in ice-cold distilled water, and used either for isolation of mitochondrial preparations or for respiratory activity measurements.

Mitochondria were isolated by the method designed in our laboratory. Cells were incubated for 30 min at room temperature in 50 mM Tris-HCl buffer, pH = 8.6, containing 10 mM dithiothreitol, washed twice with ice-cold distilled water, resuspended in 50 mM citrate phosphate buffer, pH = 5.8–5.9, containing 0.9 M sorbitol, 2 mM EDTA, 5mM $MgSO_4$, 50 mg helicase (domestically produced), and 0.5–2.0 mg zymolase (Sigma, USA) per g of original cells (wet weight), and incubated at 29 °C under mild stirring to form spheroplasts. The formation of spheroplasts was monitored by measuring the osmotic fragility of the cells by diluting, with water, small aliquots taken from the cell suspension at different times during incubation with lytic enzymes. Spheroplast formation was completed after 15 min incubation and the spheroplats were centrifugated at 400 g for 10 min and washed twice with 1.2 M sorbitol containing 0.4% BSA. The spheroplasts were homogenized with a Dounce homogenizer in a medium containing 10 mM Tris-HCl buffer, pH = 7.2, 0.4 M mannitol, 0.5 mM EDTA, 0.4% BSA. The homogenate was mixed with an equal volume of the same buffet, except that 0.4 M mannitol was substituted for 0.6 M mannitol, centrifugated for 10 min at 1200 g, and the supernatant centrifugated at 6000 g for 18–23 min. The pellets from the second spin were gently resuspended in approximately 20 mL of washing medium, (10 mM Tris-HCl buffer, pH = 7.2, 0.6 M mannitol, 0.5 mM EDTA, 0.4% BSA), recentrifugated at 6000 g for 18–23 min, and resuspended in a minimal volume of washing medium. The mitochondria thus obtained were fully active for at least 5 h when kept on ice.

Oxygen consumption of whole cells was monitored polarographically at room temperature with a Clark-type oxygen electrode in a medium containing 0.1 M potassium phosphate buffer, pH = 6.5, and 1% substrate. Respiration rates were expressed as ng-atom O consumed per milligram of dry weight. When the effects of respiratory inhibitors were measured, the incubation medium contained 1 mM KCN, an inhibitor of the cytochrome pathway, or 0.5 mM salicyl hydroxamate (SHAM), an inhibitor of the alternative oxidase. For mitochondria, the incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate, pH = 6.5, 1 mM EDTA, 20 mM Tris-pyruvate, 5 mM Tris-malate, 20 mM Tris-succinate or 4mM NADH. When indicated, 200–280 nmol ADP or 4 μg/mg protein rotenone, an inhibitor of Complex I, was added. Respiration rates were expressed as ng-atom O consumed per minute per mg of mitochondrial protein. Respiratory control was defined by the ratio of respiration in the presence of ADP (state 3) to respiration in state 4, where almsot all ADP added was phosphorylated *(13)*.

The protein content was determined by the method of Bradford *(14)* with bovine serum albumin as standard.

All experiments were run three of four times, with a good reproducibility. The values in Figs. 1 and 2 are means from two replicates ±SE. Figs. 3–6 depict typical experiments.

All chemicals used were of highest available purity and obtained from E. Merck (Germany), Sigma (USA), Fluka (Switzerland), or Serva (Germany).

Results and Discussion

At 30–43 °C, *C. blankii* cells readily utilized both fermentable and nonfermentable substrates. No significant growth was achieved at temperatures below 24 °C, thus indicating that *C. blankii* is the true thermotolerant yeast organism. Fig. 1*A* shows the kinetics of growth of *C. blankii* cells in the presence of sucrose at 30 (curve *1*) and 43 °C (curve *2*), respectively. Cells growing on sucrose at these two temperatrue regimes had generation times of 1.3–1.6 h^{-1} and growth yields (biomass synthesized per g of substrate consumed) of 0.6–0.7. Active growth at 30–43 °C was also obtained on succinate and soluble paraffins with generation times of 1.39–1.45 and 1.44–1.46, respectively.

The respiratory pattern (Fig. 1*B*) of cells grown on sucrose at 30 (curve 1) and 43 °C (curve 2) was almost identical: (1) respiratory rates reached their maxima at the mid-exponential growth phase; (2) in cells grown ex-

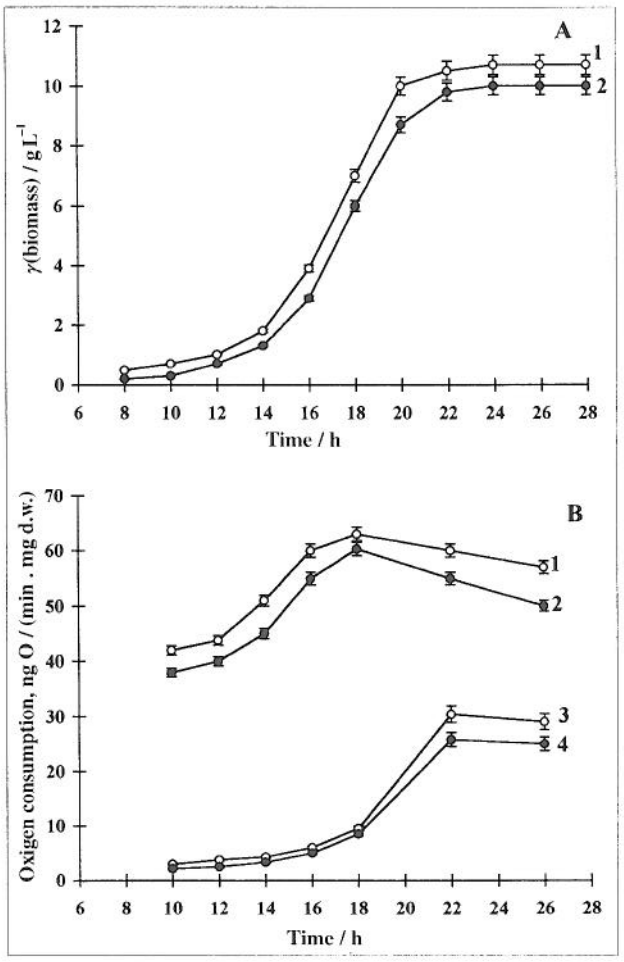

Fig. 1. Time course of biomass accumulation (A) and oxygen consumption (B) of *Candida blankii* cells grown on 1.5% sucrose at 30 (1,3) and 43 °C (2,4). 3,4 - Respiratory rates in the presence of 0.2 – 1.0 mM KCN.

ponentially, oxygen uptake was almost totally (98–100%) inhibited by 0.9 mM KCN (curves 3 and 4), indicating that the electron flux was predominantly accommodated by the cytochrome pathway; (3) in cells harvested at stationary growth phase, a considerable portion (45–60%) of respiration was cyanide-resistant, denoting the engagement of the alternative oxidative pathway. A similar respiratory pattern was observed in cells grown on sucrose or soluble paraffins (not shown).

To assess the role of the oxidative phosphorylation system in the energy budget of the cell, we examined the effect of ethidium bromide (EthBr) on cell growth. This mitochondrial mutagen has been used previously on ρ^+ cells to induce petite mutants effectively *(15–17)*. EthBr inhibits the replication of mitochondrial DNA (mit-DNA) and causes it to break into smaller molecules, whilst having no effect on nuclear DNA synthesis *(18)*. In addition, prolonged EthBr treatment converts some of the ρ^+ cells into ρ^- cells, neutral petites lacking mit-DNA *(17,19)*. Moreover, it has been shown *(20)*, that binding of ethidium bromide, a cationic dye, to mitochondrial membrane occurs at a site of low polarity and that energy conservation induces an increase in the affinity of the membrane for the dye. As some components of the oxidative phosphorylation system (apocytochrome *b*, three highest subunits of the cytochrome oxidase, one or two subunits of the ATP sythase complex, and six or seven subunits of complex I) are the products of mitochondrial translation machinery *(21)*, EthBr may be a good tool to evaluate a contribution of the respiratory system to the total energy-producing reactions in the yeast cell.

Fig. 2 depicts growth (biomass doubling) of *C. blankii* at 30 (Fig. 2A) and 43 °C (Fig. 2B) in media containing 5% glucose (fermentable substrate) (Figs. 2 A and B, curves 1) or soluble parafin (nonfermentable substrate) (Figs. 2 A and B, curves 2) as a function of EthBr concentrations. In line with the theoretical premises, low concentrations of EthBr (15 μg/mL) were found to essentially completely inhibit culture growth at 30 and 43 °C in media containing soluble paraffin, (nonfermentable substrate) (Figs. 2A and B, curves 2), indicating that glycolysis alone cannot support the growth of *C. blankii* cells, and that the mitochondrial system of oxidative phosphorylation plays a predominant role in cell supply with energy. In contrast, in glucose-containing media, glycolysis alone substantially maintained the growth at 30 °C (Fig. 2A, curve 1) and only nominally (two generations) at 43 °C (Fig. 2B, curve 1). Thus, 43 °C is still the temperature regime at which the oxidative phosphorylation system is blocked. Moreover, the higher temperature was within permissible limits (30–43 °C), the more contribution of the oxidative phosphorylation system in the energy budget of the cell was observed.

Additional information on *C. blankii* energy metabolism was obtained from the analysis of mitochondrial preparations isolated from cells exponentially grown on sucrose (Figs. 3, 4) or succinate (Figs. 5, 6) at 30 (Figs. 3, 5) and 43 °C (Figs. 4, 6), respectively.

All mitochondrial preparations were high-class quality as inferred from high respiratory rates, strong ADP control (respiratory control, RC), and ADP/O ratios close to theoretically expected maxima (3 upon oxidation

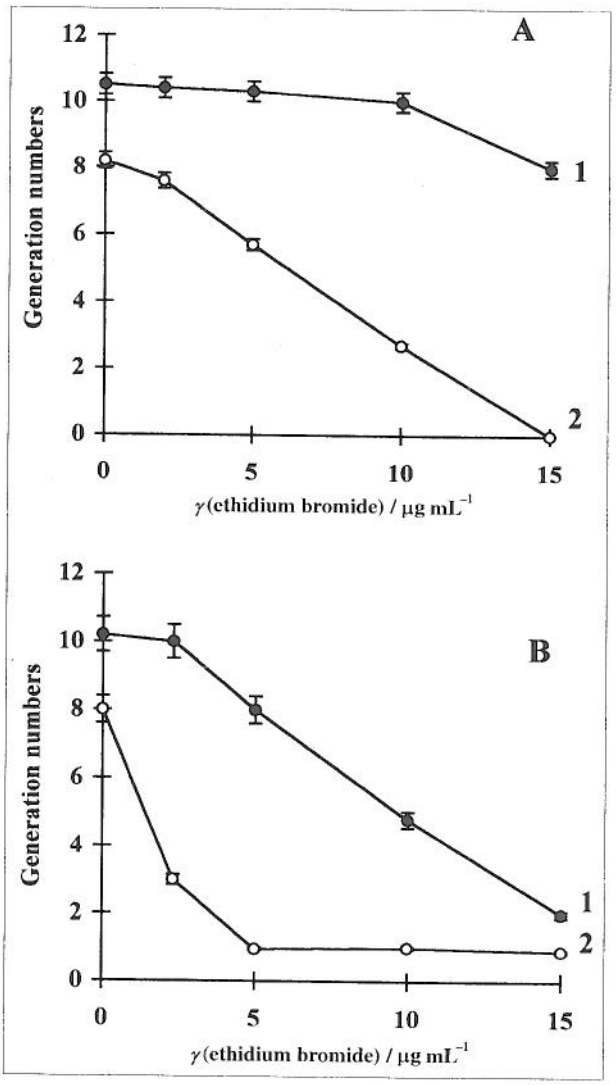

Fig. 2. Effect of ethidium bromide (EthBr) on growth of *Candida blankii* cells at 30 (A) and 43 °C (B) on 5% glucose (1) or 2% octadecane (2)

of pyruvate + malate, NAD-dependent substrates, and 2 upon oxidation of succinate or exogenous NADH, flavin-dependent substrates).

The oxidation of all substrates studied was entirely sensitive to 0.2 mM KCN (not shown). Remarkably, in mitochondria from cells of stationary growth phase, oxygen uptake was inhibited by 1 mM KCN only partly (by 75–85%, depending on the substrate used, with the succinate oxidase system being the least susceptible to cyanide), and this cyanide-resistant respiration was almost totally blocked by 0.5 mM SHAM, an inhibitor of the alternative oxidase (not shown). No conspicuous differences were found in inhibitory analysis of mitochondria from cells grown at 30 and 43 °C. The results obtained suggest that, at early growth phases, the oxidation of pyruvate + malate, succinate, and exogenous NADH proceeds exclusively via the main cytochrome respiratory pathway, while the role of the alternative pathway in the oxidation of these substrates increases with culture age (this matches the conclusion drawn earlier from the analysis of intact cell respiration, see Fig 1B).

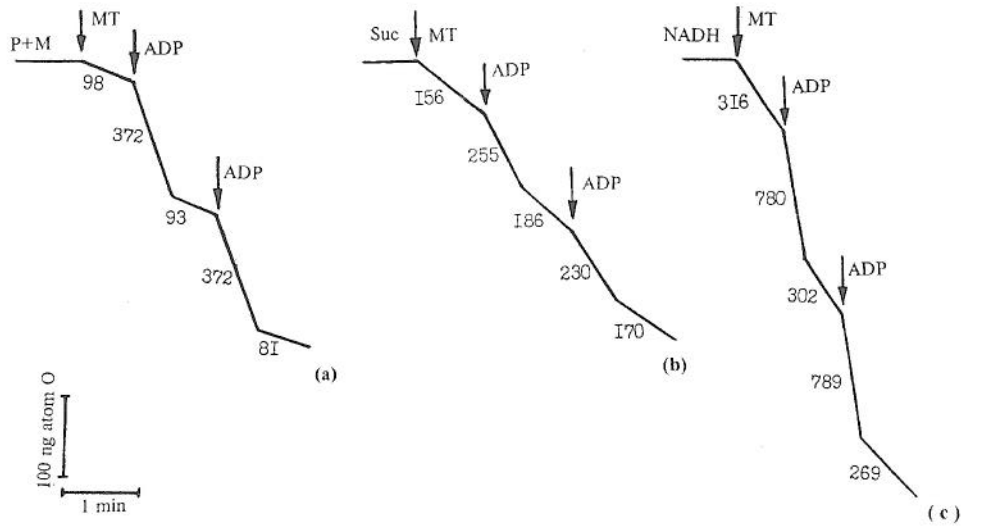

Fig. 3. Oxygen consumption by *Candida blankii* mitochondria respiring on pyruvate+malate *(a)*, succinate *(b)* and exogenous NADH *(c)*. Cells were exponentially grown on 1.5% sucrose at 30 °C. The figures along the curves indicate respiration rates (ng-atom O per min per mg of protein).
(a) – respiratory control (RC) ratios upon two successive additions of 280 nmol ADP attained 4.0 and 4.6; ADP/O ratios averaged to 2.3 and 2.4 *(b)* – RC ratios upon successive additions of 80 nmol ADP were 1.4, 1.4; ADP/O = 1.2, 1.4; *(c)* – RC ratios upon successive additions of 200 nmol ADP were 2.6, 2.9; ADP/O = 1.5, 1.5.

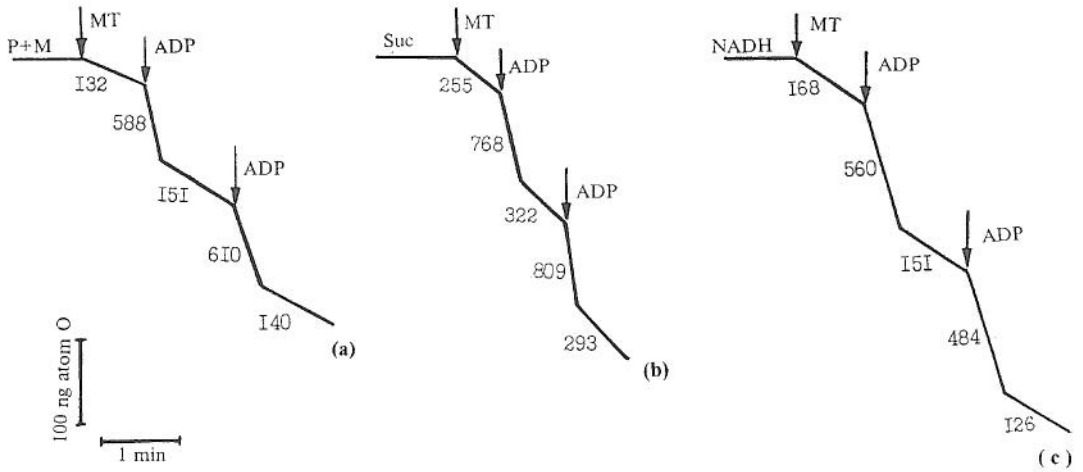

Fig. 4. Oxygen consumption by *Candida blankii* mitochondria respiring on pyruvate+malate *(a)*, succinate *(b)* and exogenous NADH *(c)*. Cells were exponentially grown on 1.5% sucrose at 43 °C. Designations as in Fig. 3.
(a) – RC = 3.9, 4.2; ADP/O = 3.0, 3.0; *(b)* – RC = 2.4, 2.8; ADP/O = 1.5, 1.5; *(c)* – RC = 3.7, 3.8; ADP/O = 1.5, 1.4.

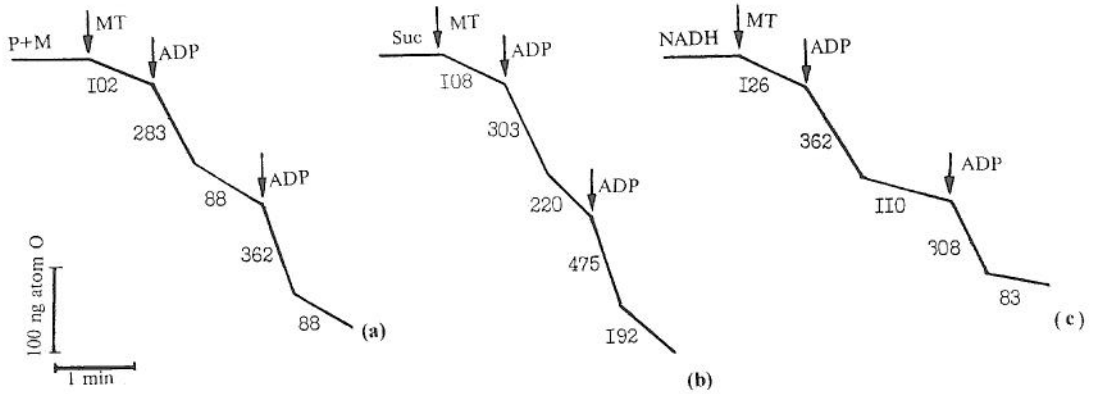

Fig. 5. Oxygen consumption by *Candida blankii* mitochondria respiring on pyruvate+malate *(a)*, succinate *(b)* and exogenous NADH *(c)*. Cells were grown on 1.5% succinate at 30 °C. Designations as in Fig. 3.
(a) – RC = 3.2, 4.1; ADP/O = 3.0, 3.0; *(b)* – RC = 1.4, 2.5; ADP/O = 1.4, 1.5; *(c)* – RC = 3.1, 3.5; ADP/O = 1.9, 2.3.

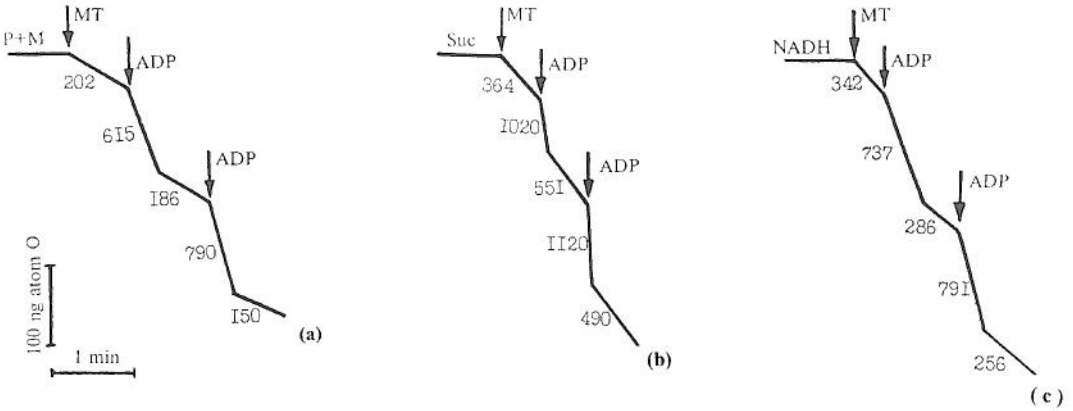

Fig. 6. Oxygen consumption by *Candida blankii* mitochondria respiring on pyruvate+malate *(a)*, succinate *(b)* and exogenous NADH *(c)*. Cells were grown on 1.5% succinate at 43 °C. Designations as in Fig. 3.
(a) – RC = 3.3, 5.3; ADP/O = 2.4, 2.3; *(b)* – RC = 1.9, 2.3; ADP/O = 1.2, 1.9; *(c)* – RC = 2.6, 3.1; ADP/O = 1.3, 1.3.

The oxidation of pyruvate + malate by mitochondria isolated from cells grown exponentially on sucrose and succinate at two temperature regimes was almost totally inhibited by rotenone (2–4 μg/mg mitochondrial protein), a potent and selective inhibitor of complex I of the respiratory chain. This, along with high ADP/O ratios upon oxidation of NAD-dependent substrates, suggests the functioning of all three points of energy conservation in the respiratory chain in exponentially growing *C. blankii* cells. All three points of energy conservations were preserved in cells harvested at stationary growth phase (not shown). It is important to recall that, in most of the studied yeast species belonging to the genus *Candida*, grown on various substrates, the first point of energy conservation was fully competent only during the stationary growth phase. During exponential growth, the oxidation of NAD-dependent substrates usually occurred, completely or partially via the rotenone-insensitive pathway, bypassing the first point of energy conservation (21,22). Thus, as distinct from most species of the genus *Candida*, in the *C. lipolytica* strain, the first point of energy conservation operated invariantly during all studied growth phases, including the earliest one. Another specific features were an increased role of the alternative terminal oxidase, especially in succinate oxidation upon transition to the stationary growth phase, retaining of active exogenous NADH oxidase at stationary growth phase (not shown), and significantly enhanced respiratory activities upon oxidation of all substrates examined by mitochondria from cells grown at 43 °C as compared to those from cells grown at 30 °C.

The main joint of this paper is that 43 °C is still the temperature regime at which the yeast respiratory chain is flourishing possibly by maintaining the barrier properties of the inner mitochondrial membrane, necessary for very efficient oxidative phosphorylation.

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References

1. P. S. Meter, J. C. Duprees, Sq Killian, *Syst. Appl. Microbiol. 15* (1992) 161–165.
2. T. Sugio, M. Takai, T. Tano, *Biosci. Biotech. Biochem. 57* (1993) 1660–1662.
3. M. W. Davis, D. D. Buechter, P. Schimmel, *Biochemistry, 33* (1994) 9904–9911.
4. T. D. Brock: *Thermophilic Microorganisms and Life at High Temperatures*, Springer, New York (1978).
5. G. Schafer, W. G. Purschke, M. Gleissner, C. L. Schmidt, *Biochim. Biophys. Acta, 1275* (1996) 16–20.
6. J. Diruggiero, F. T. Robb, R. Jagus, H. H. Klump, K. M. Borges, M. Kessel, X. H. Mai, M. W. W. Adams, *J. Biol. Chem. 268* (1993) 17767–17774.
7. R. J. M. Bergkamp, R. H. Geerse, J. M. A. Verbakel, W. Musters, R. J. Planta, *Yeast, 7* (1991) 963–970.
8. C. D. Campbell, W. J. Mchardy, *Micron, 25* (1994) 253– 255.
9. M. I. Yamani, M. M. Abujaber, *J. Dairy Sci. 77* (1994) 3558–3564.
10. N. Naganuma, Y. Usuka, K. Tanaka, *J. Gen. Appl. Microbiol. 32* (1986) 417–423.
11. K. A. G. Donald, A. Carle, M. D. Gibbs, P. L. Bergquist, *Appl. Microbiol. Biotechnol. 42* (1994) 309–312.
12. P. G. Varley, R. H. Pain, *J. Mol. Biol. 220* (1991) 531–538.
13. B. Chance, G. R. Williams, *Adv. Enzymol. 17* (1956) 65–134.
14. M. M. Bradford, *Anal. Biochem. 72* (1976) 248–254.
15. P. P. Slonimski, G. Perrodin, J. H. Croft, *Biochim. Biophys. Res. Communs, 30* (1968) 232–241.
16. G. W. Saunders, E. B. Gingold, M. K. Trembath, H. B. Lukins, A. W. Linnane, In: *Autonomy and Biogenesis of Mitochondria and Chloroplasts*, North Holland Publishing Co, Amsterdam (1971).
17. P. Nagley, A. W. Linnane, *J. Mol. Biol. 66* (1972) 181–193.
18. E. S. Goldring, L. I. Grossman, J. Marmur, *J. Bacteriol. 107* (1971) 377–381.
19. P. Nagley, A. W. Linnane, *Biochim. Biophys. Res. Communs 39* (1970) 989–997.
20. A. Azzi, M. Santano, *Biochim. Biophys. Res. Communs, 44* (1971) 211–217.
21. R. A. Zvyagilskaya, A. V. Kotelnikova, In: *Physicochem. Biol. Rev. 8* (1988) 63–109.
22. R. A. Zvyagilskaya, A. W. Kotelnikova: *Struktura i Funkzionalnaya Aktivnost' Drozhzhevykh Mitokhondrii (Structure and Functional Activity of Yeast Mitochondria)*, Nauka, Moscow (1991).

Termotolerantni soj kvasca *Candida blankii* i biokemijska svojstva s posebnim osvrtom na bioenergetiku

Sažetak

Stanice Candida blankii koriste fermentabilne i nefermantabilne supstrate pri 30–45 °C, a gornja temperaturna granica iznosi 43–45 °C. Pri temperaturi od 20 do 24 °C nije opažen pojačani rast. Način respiracije stanica koje rastu na glukozi, sukcinatu ili topljivim parafinima skoro je isti: brzina respiracije postiže svoj maksimum u eksponencijalnoj fazi rasta; u eksponencijalnoj fazi stanice su koristile samo citokromni oksidativni put; u stanica dobivenih u stacionarnoj fazi rasta postojao je istodobno citokromni i alternativni put oksidacije. Kada su stanice C. blankii rasle na topljivim parafinima, sukcinatu ili pri niskim (derepresivnim) koncentracijama glukoze, za energetske potrebe stanica koristio se sustav oksidativne fosforilacije. Dapače, što je bila viša temperatura rasta (od 30 do 43 °C), bio je veći udjel sustava oksidativne fosforilacije. Mitohondriji, izolirani iz stanica koje su rasle pri 43 °C na saharozi ili sukcinatu, imaju respiracijski lanac sa sva tri mjesta za očuvanje energije.

Otkrivene su značajne pravilnosti koje omogućavaju bolje razumijevanje rasta nižih eukariota pri visokim dopuštenim temperaturama. Vidi se da u temperaturnom području od 43 do 45 °C još uvijek djeluje respiracijski lanac, neometan mogućim promjenama propusnosti unutrašnje mitohondrijske membrane.