Pure & Appl. Chem., Vol. 65, No. 9, pp. 1893–1898, 1993.
Printed in Great Britain.

Energy balance calculations as a tool to determine maintenance energy requirements under stress conditions

L. Gustafsson, R. Ölz, K. Larsson, C. Larsson and L. Adler

Department of General and Marine Microbiology, Carl Skottsbergs Gata 22,
S-413 19 Göteborg, Sweden

Abstract
The increase in maintenance energy requirements in response to changed osmotic stress were studied in the yeast *Saccharomyces cerevisiae*. The yeast was aerobically grown at different growth rates in chemostats with glucose as the limiting substrate. The choice of growth rates served to induce different metabolisms, *i.e.* respiratory and mixed respiratory-fermentative catabolism at a low (0.09 h^{-1}) and high (0.18h^{-1}) growth rate, respectively. The total energy demand for growth at the different conditions was determined by energy balance measurements, which provided a test of the reliability of the data. Measurement of catabolic energy flux at the different growth rates and salinities (0 M and 0.9 M NaCl) were used for the calculation of specific ATP production rates. The difference in specific ATP production rate between the two salinities at the respective growth rate, indicated an additional maintenance energy requirement of 2 to 3 mmol ATP g^{-1} h^{-1} (or 0.6 - 0.8 mmol g^{-1} ks^{-1}) imposed by salt stress.

INTRODUCTION

During growth of yeast in basal medium, maintenance energy reguirements seem to be negligibly low compared to the energy requirements of reactions directly connected to growth (refs. 1-3). The requirements for maintenance are not easy (if at all possible) to determine accurately. Verdyun *et al.* (ref. 3) showed this difficulty to be due to a growth rate dependent protein content of *Saccharomyces cerevisae*, making impossible the use of the classical Pirt plot (refs. 4-6) for maintenance determinations. In more recent works (refs. 7-8), maintenance energy requirements (real and apparent) are considered both growth rate independent and growth rate dependent. Unfortunately, energy requiring metabolic processes are not easily attributed to either maintenance or anabolic, non-maintenance processes (ref. 8). Nevertheless, the maintenance energy requirements were in several studies indicated to be as low as approximately 0.5 mmol ATP g^{-1} h^{-1} (or 0.1 mmol g^{-1} ks^{-1}) for growth of different strains of *S. cerevisae* in basal medium (refs. 1-3).

During conditions of external stress, maintenance energy requirements may increase considerably. When *S. cerevisiae* was cultivated in a chemostat in medium containing 1 M NaCl, the maintenance energy requirements increased four-fold compared to growth in basal medium (ref. 1). Likewise, by decreasing the environmental pH from 5 to 3 caused at least an eight-fold increase in maintenance energy requirements in yeast (ref. 3). During aerobic batch cultivations, a considerable increase in maintenance energy requirements was indicated both at low external pH and low osmotic potential for the yeasts *S. cerevisae* and *Debaryomyces hansenii* (ref. 9). However, since cells in carbon- and energy-rich media (batch cultivations) may not adjust to minimum energy demands (ref. 10), maintenance energy requirements may be greatly overestimated during such conditions. The studies by Watson (ref. 1) and Verduyn *et al.* (ref. 3) were, on the other hand, performed by chemostat cultivations under strictly fermentative conditions, using a respiration-deficient mutant of *S. cerevisiae* or anaerobic conditions, respectively. In addition to providing energy-limitation, these approaches also circumvented the difficulty imposed by a mixed respiratory-fermentative metabolism during growth. To be able to study the increased energy requirements for maintenance during aerobic growth of *S. cerevisiae* under an increased work load caused by external stress, we took a different approach (ref. 11). In summary, *S. cerevisiae* was grown in a chemostat under aerobic conditions at different dilution rates and salinities. Absolute maintenance energy requirements were not determined. Instead, the *additional* maintenance energy requirements imposed by an increased external salinity were estimated at different growth rates (= dilution rates). The range of dilution rates chosen resulted in a shift from a purely respiratory to a mixed respiratory-fermentative catabolism. Energy balances were used to determine the increased maintenance energy requirements at 0.9 M NaCl compared to growth in basal medium. The present paper describes in detail in which way these calculations were performed.

MATERIALS AND METHODS

The yeast strain used was *Saccharomyces cerevisae* Y41 (ATCC 38531; ref. 12). The medium consisted of nitrogen base (YNB) without amino acids (Difco) and was supplemented with sterile glucose after autoclaving to a final concentration of 0.5% (w/v). For experiments at a reduced external osmotic potential, NaCl was added to a final concentration of 0.9 M. The experimental design, substrate and product analyses, as well as microcalorimetric measurement were as previously described (ref. 11).

THEORY AND CALCULATIONS

Two approaches were used for calculating *additional maintenance energy requirements* at 0.9 M NaCl compared to a reference state (0 M NaCl) during aerobic growth of *S. cerevisiae*:

(*a*) Energy balances were calculated to determine the total amount of energy consumed for steady state growth in a chemostat at various growth rates in media containing 0 M or 0.9 M NaCl. The energy calculations should yield the same result whether performed on the total amount of substrates used or on the total amount of products formed (including heat), enabling a test of the reliability of the data. The standard molar enthalpies used (ref. 13) for substrates and products refer to their aqueous state, except for gases and biomass. Gases (CO_2, O_2) were considered to be in their gaseous state under the actual experimental conditions, while standard enthalpies for hydrated, live biomass are not known. Recent experiments (ref. 13) indicate that the enthalpy of transition from "wet" biomass to dry biomass is negligible. Therefore, enthalpy values of dry biomass have been used in this study. Since the combustion products of all components have been used as the reference state, water (liquid), carbon dioxide (gaseous), oxygen (gaseous), and protons (aqueous) were omitted from the calculations. The enthalpy of combustion of the dry biomass, the elemental composition (used for the calulations described below) and the ash and water content of the dry biomass were determined at the Institute of Chemical Engineering, Lausanne, Switzerland by the method of Gurakan *et al.* (ref. 14). The enthalpies of combustion were -21.7 kJ g^{-1} and -22.3 kJ g^{-1} of ash free biomass for cells grown at 0 M and 0.9 M NaCl, respectively.

(*b*) In re-calculating the additional energy requirements for growth at 0.9 M as compared to 0 M NaCl as a difference in ATP turn-over per g biomass and hour, the proportion of anabolism to catabolism and the different catabolic subreactions, including respiratory and fermentative metabolism have to be determined. In the study by Ölz *et al.* (ref. 11), the consumption of the carbon- and energy-source (glucose) was determined as were all the dominating organic products (biomass, ethanol, glycerol and acetate). Since oxygen consumption and carbon dioxide production were not measured, growth equations were constructed by using the concept of degree of reduction (refs. 15-17). The degree of reduction ($\gamma_i = 4C + H - 2O - 3N$, where C, H, O and N denote the atomic coefficient of the elements of a C-molar compound, i, in which C = 1) expresses the number of available electrons per unit carbon atom. This expression of the degree of reduction includes the factor -3N, which compensates for the electrons donated to the biomass from ammonia and thereby omits ammonium consumption from the calculations. For the construction of growth equations, the elemental composition of the biomass was determined (ref 14; see above) to $CH_{1.64}O_{0.55}N_{0.16}$ and $CH_{1.66}O_{0.55}N_{0.19}$ for cells cultivated with and without NaCl, respectively. In establishing growth equations, subreactions for which the products (*i.e.* biomass, ethanol and glycerol) are more reduced than the substrate (glucose) were balanced by CO_2 production (ref. 18; *cf.* ref. 17). The biochemical relevance of the anabolically produced CO_2, is due to the NADPH requirement for biosynthesis. The NADPH generation via the pentose phosphate pathway will result in CO_2 production. Formation of glycerol requires NADH, provided by biomass production or products such as acetate, which also is accompanied by carbon dioxide formation (refs. 19-20). The proportion of the substrate (glucose) used for respiration was attained as the difference between the total amount of substrate consumed and the sum of glucose used for biomass, ethanol, glycerol, acetate and carbon dioxide production accompanying the formation of biomass and ethanol. In addition, the carbon dioxide used for redox balancing the glycerol production (as calculated by the degree of reduction) was included, while the carbon dioxide formation accompanying the acetate production was of the same reason omitted from the calculations.

The growth equation attained for growth of *S. cerevisae* in 0.9M NaCl at a dilution rate of 0.09 h^{-1} will be used as an example:

$$\alpha,\beta\text{-}DC_6H_{12}O_6(aq) + 0.333\ NH_4^+(aq) + 2.764\ O_2(g) \longrightarrow 2.080\ CH_{1.64}O_{0.55}N_{0.16} + 0.333\ H^+(aq)$$

$$+ 2.910\ CO_2(g) + 3.558\ H_2O(l) + 0.226\ C_3H_8O_3(aq) + 0.166\ C_2H_4O_2(aq) \qquad \text{(Eq. 1)}$$

The respiratory contribution to Eq. 1 is:

$$0.461\ \alpha,\beta\text{-}DC_6H_{12}O_6(aq) + 2.764\ O_2(g) \longrightarrow 2.764\ CO_2(g) + 2.764\ H_2O(l) \qquad \text{(Eq. 2)}$$

During aerobic growth, ATP production will result both from (*A*) *substrate level phosphorylation* and (*B*) *electron transport phosphorylation*:

(*A*) *Substrate level phosphorylation*: The ATP production per unit of biomass formed ($Y_{ATP/X}$; mol per g) can be calculated directly from (*i*) the organic product formation (excluding biomass) plus (*ii*) the substrate level phosphorylation in the TCA cycle according to:

$$(i) \qquad Y_{ATP/X}\ (mol/g) = \frac{Y_{E/G} + Y_{A/G} - Y_{Gly/G}}{Y_{X/G}} \qquad (mol/g)$$

$$(ii) \qquad Y_{ATP/X}\ (mol/g) = \frac{(2/3)(Y_{O2/G})}{Y_{X/G}} \qquad (mol/g)$$

(E=Ethanol, A=Acetate, Gly=Glycerol, O_2=Oxygen, G=Glucose and X=Biomass)

(*B*) *Electron transport phosphorylation*: Since the P/O-ratio for the different salinities and growth rates are not known, a constant P/O-ratio of 1.0 was assumed for all conditions (refs. 3 and 21). ATP will be produced in direct proportion to the production of reducing equivalents (NADH and FADH). As can be seen in Figure 1, the ATP production via oxidation of reducing equivalents and electron transport phosphorylation is:

$$Y_{ATP/X}\ (mol/g) = \frac{2(Y_{A/G}) - Y_{Gly/G} + 2(Y_{O2/G})}{Y_{X/G}} \qquad (mol/g)$$

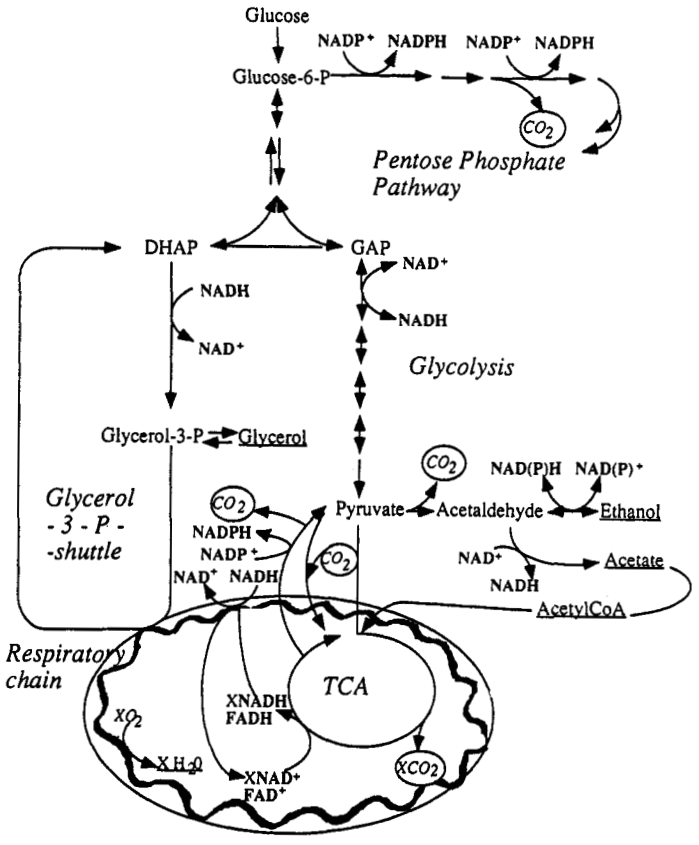

Figure 1. *Simplified scheme of intermediary metabolism of S. cerevisiae. For details see refs. 22 and 23. Redrawn from ref. 18.*

The term ($Y_{O2/G}$ x 2) includes production of 2NADH in the glycolysis and 8NADH plus 2FADH in the TCA cycle per mole of glucose consumed. Since NADH produced in the cytoplasm can be reduced via an "external NADH dehyrogenase" on the outer surface of the inner mitochondrial membrane (or via the

glycerol-3-P-shuttle) and since the proton translocating site at neither the level of the internal nor external mitochondrial NADH dehydrogenase seems to be used in growing *S. cerevisiae* (ref. 24), all NADH and FADH produced will, with an assumed P/O-ratio of 1.0, result in 1 ATP each.

Taking respiratory growth (dilution rate (D) = 0.09 h^{-1}) of *S. cerevisae* in a medium containing 0.9 M NaCl (Eq. 1 and 2) as an example, the total rate of ATP production per unit of biomass $D(Y_{ATP/X})$ is:

$$D(Y_{ATP/X}) = \frac{1.783^{a} + 5.634^{b}}{55.73^{c}} \times 0.09 = 12.0 \text{ mmol } g^{-1} h^{-1} \text{ (or } 3.3 \text{ mmol } g^{-1} ks^{-1})$$

ATP production via [a]substrate level phosphorylation and [b]electron transport phosphorylation.
[c]The growth yield (g dry biomass formed/mol glucose consumed; ash-containing biomass).

RESULTS AND DISCUSSION

S. cerevisiae invests an increased proportion of its energy source into reactions other than biosynthesis when cultured under salt stress (refs. 1, 9 and 11). This increased energy expenditure during growth in saline environments is characterized primarily by (*i*) an increased maintenance energy and (*ii*) an increased production of glycerol. The intracellular level of glycerol, which increased dramatically with salinity, was maintained independent of growth rate and type of catabolism (ref. 11) consistent with the role of glycerol as a compatible solute in yeasts (ref. 25). At an external concentration of 0.9 M NaCl, the internal glycerol concentration was kept at about 1.2 mol kg^{-1} solvent (molal). However, the total glycerol production increased considerably with growth rate in the salinity medium, which was also the case of maintenance energy requirements imposed by salt stress (ref. 11). The increased energy used for maintenance in response to salt stress may largely be explained by a requirement for enhanced ion pumping to minimize the impact of the external salinity on the intracellular physiology. The intracellular K^+ was kept around 0.29 molal irrespective of external salinity, while the internal Na^+ concentration increased from about 0.02 molal in basal medium to about 0.18 molal in 0.9 M NaCl medium (ref. 11). These data support previous findings (ref. 26) that Na^+ is relatively effectively excluded from the cells against an unfavourable concentration gradient over the plasma membrane.

Including the energy conserved in glycerol, the total additional energy demand for growth at 0.9 M NaCl increased with growth rate and corresponded to 28 and 51% of the energy required for growth at 0M NaCl at D= 0.09 and 0.18 h^{-1}, respectively. On the basis of total substrate consumption, the additional maintenance energy expenditure for growth at 0.9 M NaCl can be calculated, from data given in ref. 11, to increase from -150 mW per g biomass to -1100 mW per g with an increase in growth rate from 0.09 to 0.18 h^{-1}, respectively. The higher value is similar to that noted for the additional energy expenditure for growth of *S. cerevisiae* at 0.7 M NaCl in batch culture (ref. 9) and in agreement with the hypothesis that energetic efficiency is considerably lower during energy excess than during energy limitation (refs. 10, 27-28).

The data presented so far were performed as described under (*a*) in "Theory and Calculations" and are presented and discussed in ref. 11. We have in this report extended the presentation to include the calculation of ATP production from the same data (see (*b*) in "Theory and Calculations"). In Figure 2 the net production rate of ATP, as well as its positive (ATP yielding) and negative (ATP consuming) components are given for growth at different salinities (0 and 0.9 M NaCl) and dilution rates (D=0.09 and 0.18h^{-1}). The choice of dilution rates represent respiratory and mixed respiratory-fermentative growth of *S. cerevisiae*, respectively. Although as much as 53% (36 kJ per g ash free biomass formed) and 39% (41 kJ per g) of the total carbon consumption was diverted to ethanol fermentation in the low and high salinity, respectively, at D=0.18h^{-1} (ref. 11), respiration largely dominated in terms of ATP production also during the respiro-fermentative metabolism (Fig. 2). The glycerol production constitutes an ATP drainage, which increased with salinity due to the osmoregulatory role of glycerol.

ATP was produced both via substrate level phosphorylation and electron transport phosphorylation. Only the former is used during ethanol fermentation, while the NADH production resulting from complete glucose oxidation and acetate formation leads to ATP generation both from substrate level phosphorylation and from electron transport phosphorylation. Glycerol production, on the other hand, not only consumes ATP directly but also indirectly through consumption of NADH. *S. cerevisiae* has at least two possibilities of ATP generation from NADH formed in the cytoplasm, *i.e.* via the "external mitochondrial NADH dehydrogenase" and the glycerol-3-P pathway (Fig. 1). In addition, ATP production may result from electron transport phosphorylation because of surplus NADH generation during assimilation (ref. 19), the contribution of which can be calculated from the cellular composition (ref. 29). By this approach Verduyn *et al.* (ref. 20) calculated the surplus generation of NADH to be 1.4 mmol per g biomass and h (or 0.4 mmol g^{-1} ks^{-1}), at a dilution rate of 0.10 h^{-1}. Using the data of Verduyn *et al.* (ref. 20), we calculated a surplus NADH production rate of approximately 1.6 mmol g^{-1} h^{-1} (or 0.4 mmol g^{-1} ks^{-1}) at D=0.18 h^{-1}. The observed increase in assimilatory NADH generation with growth rate, is

mainly explained by an increasing cellular protein content with increasing growth rates. Assuming a P/O-ratio of 1.0 (refs. 3 and 21), the assimilatory NADH generation will result in a specific ATP production rate of 1.4 and 1.6 mmol g^{-1} h^{-1}, respectively. This ATP production is, however, already compensated for by calculating according to (b) in "Theory and Calculations", since the *net* need for reducing equivalents for biomass production is accounted for by redox-balancing, using the degree of reduction. The rest of the assimilatory related surplus of reducing equivalents is included in the calculated respiratory part.

An uncertain assumption in the calculations of ATP production presented above is the value of the P/O-ratio, which in all our calculations was assumed to be 1.0 (refs. 3 and 21). Only small differences were shown between P/O-ratios of the mitochondrial NADH dehydrogenases facing the cytoplasm or the mitochondrial matrix (ref. 24). In addition, a maximum P/O-ratio of about 2 is reported for growing *S. cerevisiae*, since the electron transport chain lacks the first proton translocationg site. Consequently, all NADH and FADH oxidized in the respiratory chain were considered to result in the same P/O-ratio. However, it is possible that our assumption of a constant P/O-ratio may result in overestimated ATP-values at high growth rates, since a decreasing P/O -ratio was obtained at an increasing respiratory flux with isolated mitochondria from *S. cerevisiae* (ref. 24).

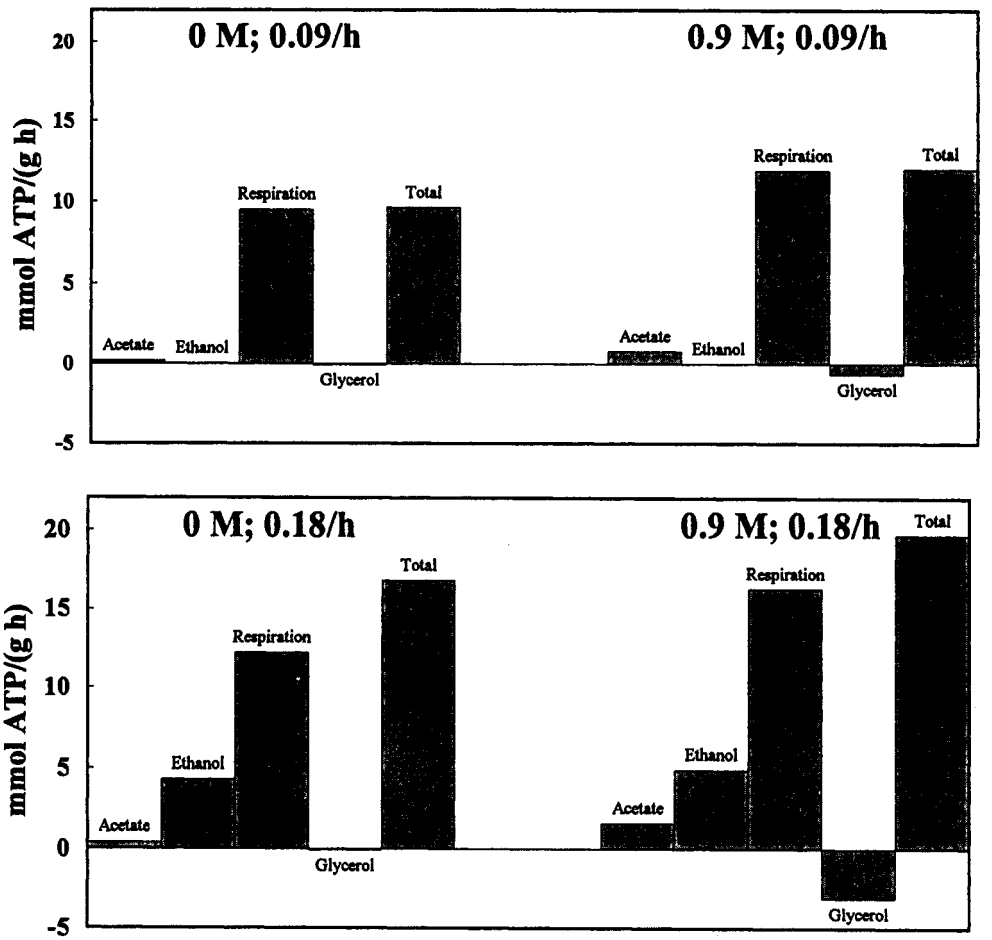

Figure 2. Specific rates of the total ATP production and of ATP yielding (acetate and ethanol production as well as respiration) and ATP consuming (glycerol production) catabolism during growth of S. cerevisiae at 0 M and 0.9 M NaCl and at two different dilution rates: 0.09 h^{-1} (the upper) and 0.18 h^{-1} (the lower).

Anyhow, the difference between the *net* specific rate of ATP production at the different external salinities indicated an additional salt imposed maintenance energy requirement of 2.4 and 2.9 mmol ATP g^{-1} h^{-1} (or 0.7 and 0.8 g^{-1} ks^{-1}) at a dilution rate of 0.09 and 0.18 h^{-1}, respectively (Fig. 2). This additional energy requirement for maintenance can at least partly be explained by increased ion pumping demands at an increased external salinity. However, whether the efficiency of energy transformations remains the same, for example resulting in equal P/O-ratios, for cells grown under salt stress as compared to basal medium will be a central focus in our further studies.

Acknowledgements

This study was supported by grants from the Swedish National Board for Natural Sciences, the Swedish National Board for Technical Development, and the Carl Trygger Foundation. R.Ö. was supported by scholarships from the Swedish Institute and the Bundesministerium fur Wissenschaft und Forschung of Austria. Many thanks to Drs. Erich Gnaiger and Richard Kemp for helpful comments.

REFERENCES

1. T.G. Watson, J. Gen Microbiol. 64, 91-99 (1970).
2. P.J. Rogers and P.R. Stewart, Arch. Microbiol. 99, 25-46 (1974).
3. C. Verduyn, E. Postma, W.A. Scheffers and J.P. van Dijken, J. Gen. Microbiol. 136, 405-412 (1990).
4. S.J. Pirt, Proc. R. Soc. Lon. 163B, 224-231 (1965).
5. A.H. Stouthamer, Antonie van Leeuwenhoek 39, 545-565 (1973).
6. A.H. Stouthamer and C.Bettenhaussen, Biochim. Biophys. Acta 301, 53-70 (1973).
7. S.J. Pirt, J. Ferment. Technol. 65, 173-177 (1987).
8. B.A. Bulthuis, G.M. Koningstein, A.H. Stouthamer, and H.W. van Verseveld, Arch. Microbiol. 152, 499-507 (1989).
9. L. Gustafsson and C. Larsson, Thermochim. Acta 172, 95-104 (1990).
10. D.W. Tempest and O.M. Neijssel, Annu. Rev. Microbiol. 38, 459-486 (1984).
11. R. Ölz, K. Larsson, L. Adler and L. Gustafsson, J. Bacteriol. 175 (1993), in press.
12. J.C. Anand and A.D. Brown, J. Gen. Microbiol. 52, 205-212 (1968).
13. U. von Stockar, E. Gnaiger, L. Gustafsson, C. Larsson, P. Tissot, and I. Marison, submitted for publication.
14. T. Gurakan, I.W. Marison, U. von Stockar, L. Gustafsson and E. Gnaiger, Thermochim. Acta 172, 251-266 (1990).
15. I.G. Minkevich and V.K. Eroshin, Studia. Biophys. Berlin 49, 43 (1975).
16. J.A. Roels, Energetics and Kinetics in Biotechnology, Elsevier Biomedical press, Amsterdam (1983).
17. E.H. Battley, Energetics of Microbial Growth, John Wiley & Sons, New York (1987).
18. R. Ölz, K. Larsson, C. Larsson, E. Gnaiger and L. Gustafsson, in Modern Trends in Biothermokinetics (J.P. Mazat, S. Schouster and M. Rigoulet, eds.), Plenum Press (1993), accepted.
19. J.P. van Dijken and W.A. Scheffers, FEMS Microbiol. Rev. 32, 199-224 (1986).
20. C. Verduyn, Energetic Aspects of Metabolic Fluxes in Yeasts, Thesis, The Delft University of Technology, The Netherlands (1992).
21. C. Verduyn, A.H. Stouthamer, W.A. Scheffers and J.P. van Dijken, Antonie van Leeuwenhoek 59, 49-63 (1991).
22. D.G. Fraenkel, In: The Molecular Biology of the yeast *Saccharomyces*: Metabolism and Gene Expression, N.J. Strathern, E.W. Jones and J.R. Broad (eds.), pp. 1-37, Cold Spring Harbor Laboratory, Cold Spring Harbor, NewYork (1982).
23. C. Wills, T. Martin and T. Melham, Arch. Biochem. Biophys. 246, 306-320 (1986).
24. R. Ouhabi, M. Rigoulet and B. Guerin, FEBS Lett. 254, 199-202 (1989).
25. A. Blomberg and L. Adler, Adv. Microb. Physiol. 33, 145-212 (1992).
26. B. Norkrans and A. Kylin, J. Bacteriol. 100, 836-845 (1969).
27. H. Westerhoff, K.J. Hellingwerf and K. van Dam, Proc. Natl. Acad. Sci. USA 80, 305-309 (1983).
28. E. Gnaiger, In: Surviving Hypoxia: Mechanisms of Control and Adaptation, P.W. Hochachka, P.L. Lutz, T. Sick, M. Rosenthal and G. Van den Thillart (eds.), pp. 77-109, CRC Press, Florida (1993).
29. C. Verduyn, E. Postma, W.A. Scheffers and J.P. van Dijken, J. Gen. Microbiol. 136, 395-403 (1990).