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ENERGY BUDGETING IN STUDYING THE EFFECT OF ENVIRONMENTAL FACTORS ON THE ENERGY METABOLISM OF YEASTS *

L. GUSTAFSSON and C. LARSSON
Department of General and Marine Microbiology, University of Göteborg,
Carl Skottsbergs Gata 22, S-413 19 Göteborg (Sweden)

SUMMARY

The yeast *Debaryomyces hansenii*, ubiquitous in marine environments and known to be highly osmotolerant, showed a higher efficency in coping with increased salinities in comparison to baker's and brewer's yeast *Saccharomyces cerevisiae*. Both yeasts changed their catabolic flows in response to increased salinities by producing glycerol, which partly explains the decreased growth yields at increased salinities. *S.cerevisiae*, on the other hand, demonstrated a higher efficiency at low pH values in comparison to *D.hansenii*. *S.cerevisiae* did not change its size-distribution of metabolic flows until a very low pH was reached, while *D.hansenii* showed higher heat yields and an increased arabinitol production in response to a more moderate decrease in pH.

INTRODUCTION

The words "yeast" and "fermentation" are traditionally closely associated. However, the majority of all known yeast species use a respiratory metabolism and lack entirely or almost entirely the capacity to ferment. The respiratory capacity endows many yeasts with the ability to metabolize a large spectrum of organic compounds and thereby expands their ability to occupy different ecological niches (ref. 1). The capacity to use a fermentative and/or a respiratory metabolism may be explained by the natural habitats of yeasts. For example, the predominant yeasts isolated from open ocean waters, including the ubiquitous species *Debaryomyces hansenii*, are typified by respiratory metabolism (ref. 2-3), while yeasts possessing the capacity to ferment, including baker's and brewer's yeast *Saccharomyces cerevisiae*, have been isolated from nutrient-rich environments (ref. 1,4).

Yeast's broad tolerance to changes in environmental factors, such as in water potential (ref. 4-5) and pH (ref. 4) also expands their range of possible habitats for survival and growth. The highly osmotolerant marine species *D.hansenii* is capable of growth in media containing up to about 4 M NaCl (refs. 6-7). *S.cerevisiae*, referred to as a non-osmotolerant yeast, still grows in media containing up to 1.7 M NaCl (ref. 6). Regarding the pH-range, yeast-growth can be found from below 2.0 to about 9.0 (ref. 4). Although yeasts may tolerate for example low water-potentials and pH-values, growth at such extremes is often accompanied by reduced yields, and prolonged adaption periods and generation times (ref. 7).

The aim of this study was to compare the effect of different pH ranges and water potentials on the energy flows and budgets of different energy metabolisms during growth of the yeasts *D.hansenii* and *S.cerevisiae*.

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MATERIALS AND METHODS

Yeast strains, media and growth conditions

S.cerevisiae strain Y 41 (ATCC 38531)(ref. 8) and *D.hansenii* (Zopf) van Rij, strain 26 (ref. 7) were maintained on nutrient Wickerham agar (ref. 9). *D.hansenii* was cultured in a defined liquid medium (ref. 10), which included urea (0.4 g/l) as the nitrogen source. Where stated, the nitrogen source was changed to sterile-filtered (0.22 μm, Millipore) urea (0.4 g/l) added after autoclaving to avoid ammonium formation, sterile-filtered urea (0.4 g/l) plus $(NH_4)_2SO_4$ (0.12 g/l), or $(NH_4)_2SO_4$ (1.0 g/l). Glucose (0.5 %, wt/vol) was used as the carbon- and energy source. For experiments with *S.cerevisiae*, yeast nitrogen base without amino acids (YNB)(Difco) was used. The YNB-medium was supplemented with glucose or sterile filtered ethanol as the carbon- and energy source at a final concentration of 0.5 % (wt/vol). For the salt stress experiments, the media were supplemented with NaCl to give a final concentration for *S.cerevisiae* of 0.34 or 0.68 M and for *D.hansenii* of 1.35 M. Cultures without pH-adjustment had an initial pH of 4.5 (*S.cerevisiae*) or around 6 (*D.hansenii*), while the pH of pH-adjusted cultures was automatically controlled to pH 4.5 or 6.0, respectively, by the addition of 0.5 M NaOH. Fed-batch cultures of *S.cerevisiae* were grown at a controlled pH of 4.5 in a fermentor (LKB 1601 Ultroferm) with operating conditions essentially as previously described (ref. 11). Glucose was added in portions of 0.2 g/l to a total addition of 0.5 or 1 % (wt/vol). The additions were calorimetrically controlled. Inoculum cultures were grown aerobically in liquid medium, supplemented with 1 % (wt/vol) glucose, with mechanical stirring to the stationary phase (3 days). A 10-ml inoculum was added to 250 ml of medium in a conical 1 l flask to give an initial cell concentration of about 0.1 g dry weight/l (*S.cerevisiae*) or about 0.2 g dry weight/l (*D.hansenii*). All liquid cultures were incubated in a water bath at 30°C, or 27°C where stated, and stirred mechanically. In all experiments, the rate of heat production (μW) was monitored continuously. Samples were withdrawn at selected intervals. Growth was followed turbidometrically at 610 nm, and optical density values were used to yield dry weight values as previously described (ref. 12).

Microcalorimetry

The heat production rate (dQ/dt) was measured with a multichannel microcalorimeter (Bioactivity Monitor LKB 2277) of the heat conduction type (ref. 13), equipped with flowthrough cells. In addition to external electrical calibration, internal calibration was performed with a chemical reaction (ref. 14). The calorimetric experiments were run as previously described at 27°C (ref. 12) or at 30°C (ref. 15).

Determinations of substrate and metabolite concentrations

Determinations of substrate and metabolite concentrations were performed as previously described for glucose, ethanol, glycerol and acetate (ref. 15) or for glycerol determinations for experiments run at 27°C (ref. 12). Total polyol and arabinitol concentrations were determined according to Adler and Gustafsson (ref. 10).

Determinations of intracellular potassium content and cell volume

The procedures used for determinations of intracellular potassium content and cell volume have been described by Larsson *et al.* (ref. 16).

Elemental and heat of combustion analyses

The elemental and heat of combustion analyses were performed at the Institute of Chemical Engineering, Swiss Federal Institute of Technology, Lausanne, Switzerland, mainly by the method described by Cordier *et al.* (ref. 17). Further details are given by Larsson (ref. 18). These analyses were used in energy recovery (ER) calculations. Energy recoveries are expressed as the sum of the energy content of the products formed divided by the sum of the energy content in the substrates used. For the energy recovery calculations, the products of combustion have been used as the reference state and the actual thermodynamic state (aqueous, gaseous etc.) has been used for the reactants and products. The heat of combustion values obtained were -21.39 ± 0.33 kJ/g_{af} dry weight (SD, 4n) and -21.76 ± 0.20 kJ/g_{af} dry weight (SD, 3n) for *S.cerevisiae* and *D.hansenii*, respectively.

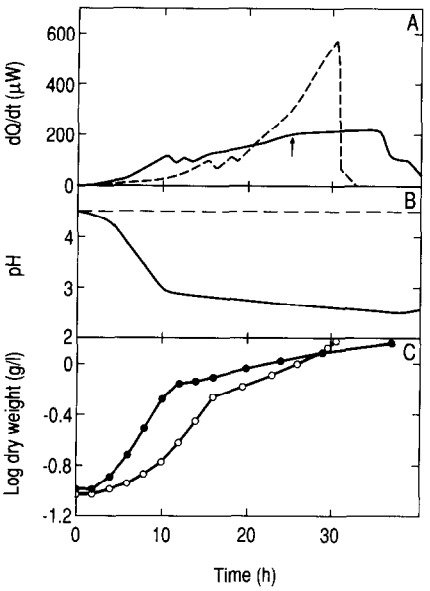
RESULTS

The effect of pH on growth of *S.cerevisiae* and *D.hanseenii*

The change in pH from 4.5 to 2.5 during growth of non-pH adjusted cultures of *S.cerevisiae* strongly affected growth as compared to pH adjusted cultures (pH 4.5) (Fig. 1). The metabolic rate during the respiro-fermentative phase, *i.e.* the phase resulting from a mixed respiratory and fermentative catabolism of glucose and continuing to the first maximum (start of the transition phase) in the heat production rate curve (ref. 15), was highest in cultures where pH was reduced during growth. In contrast, the highest metabolic rate was attained in pH adjusted cultures during the respiratory phase, where the ethanol produced in the respiro-fermentative phase is respired, and which lasted from about the second minimum to the highest maximum in the heat production rate curve (ref. 15).

Although the rates of the metabolic flows were affected by the change in pH, the size-distribution of the metabolic flows was not significantly changed, as exemplified in Figure 2, except for the latter part of the respiratory phase where the heat yield increased simultaneously with a pH reduction below 2.8 (Fig. 1A, B). This did not however, markedly influence the yield of the total growth (Fig. 1C).

When the growth medium of *D.hansenii* contained a mixture of ammonium and urea, the microcalorimetric monitoring indicated a metabolic shift between 7 and 10 hours of cultivation, as reflected by oscillations in the heat production rate curve (Fig. 3A). This was due to the exhaustion of the preferred nitrogen source, ammonium, and the initiation of growth on the secondary nitrogen source, urea (ref. 18). During growth on ammonium there was an efflux of one proton for each nitrogen conserved in biomass (ref. 18), causing a reduction in pH (Fig. 3B). The metabolic rates of *D.hansenii* were strongly affected by a change in the pH range during growth (Fig. 3) as for *S.cerevisiae*, but contrary to *S.cerevisiae*, a reduction in pH below 4 did change the size-distribution of the flow of metabolites, as shown by the reduction in



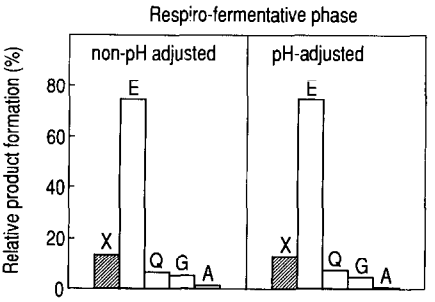


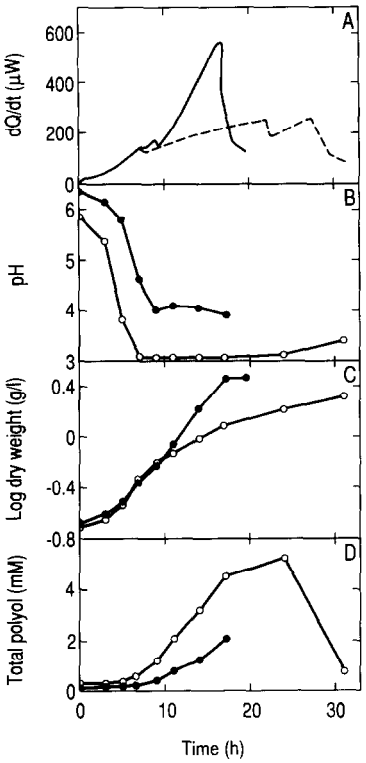
Fig. 1. (A) Rate of heat production, dQ/dt, (B) pH and (C) dry weight during growth of *S.cerevisiae* in pH adjusted (broken lines and open circles) and non-pH adjusted cultures (full lines and closed circles). The arrow denotes a shift in heat yield during the respiratory phase of the non-pH adjusted culture, see text for explanation.

Fig. 2. The relative product formation, calculated as the energy content of each product (kJ/l) in percentage of the total energy content of the products (kJ/l) of a non-pH adjusted and a pH adjusted culture of *S.cerevisiae* in the respiro-fermentative phase. X = Biomass; E = Ethanol; Q = Heat; G = Glycerol; A = Acetate.

TABLE 1
Specific growth rates (μ) and heat yields (ΔQ_X; kJ heat dissipated/g biomass formed) for *D. hansenii* during different external pH ranges.

Nitrogen source	Growth phase	pH-range	μ (h^{-1})	ΔQ_X (kJ/g)
Urea[a]	exponential urea growth	6.1-5.2	0.243±0.025 (SD, 8)	-12.3±1.1 (SD, 8)
Urea and ammonium	exponential urea growth	4.5-3.7	0.207±0.040 (SD,11)	-12.2±1.9 (SD,11)
Urea and ammonium	exponential urea growth	3.6-3.5	0.166±0.018 (SD, 3)	-15.8±0.3 (SD, 3)
Urea and ammonium[b]	exponential urea growth	3.1-3.0	0.074±0.026 (SD, 4)	-28.6±2.8 (SD, 4)

[a] Sterile-filtered urea (0.4 g/l)
[b] Sterile-filtered urea (0.4 g/l) plus $(NH_4)_2SO_4$ (0.12 g/l)
Variation as standard deviation; number of observations in brackets.



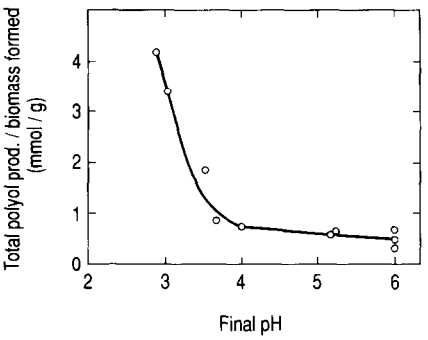


Fig. 3. (A) Rate of heat production, dQ/dt, (B) pH, (C) dry weight and (D) total polyol production (arabinitol), expressed as mmoles per l of culture during growth of *D.hansenii* with urea (0.4 g/l, which is partly degraded in the autoclave, resulting in an ammonium concentration of 0.04 g/l) (full line and closed circles), and sterile-filtered urea (0.4 g/l) plus $(NH_4)_2SO_4$ (0.12 g/l) (broken line and open cirlces) as the nitrogen source.

Fig. 4. Total polyol production (arabinitol) per unit of biomass formed in relation to the extracellular pH during growth of *D.hansenii*. The different external pH were attained by pH adjustment or the use of different nitrogen sources or mixtures of nitrogen sources (see Material and Methods and Table 1 for explanation).

growth yield (Fig. 3C), the increased polyol production (Fig. 3D) and the increased heat yield, *i.e.* the heat production per unit of biomass formed (Table 1). The polyol pool consisted entirely of arabinitol. The amount of polyol produced per biomass formed was about 0.7 mmoles/g as long as the pH was above 3.7 (Fig. 4). When the pH was reduced below this value there was a sharp increase in arabinitol production, with values of about 4 mmoles per g biomass formed at a pH of 2.9. Below this pH growth stopped, but the arabinitol production continued to values of about 7 mmoles per g biomass formed. There was a simultaneous depletion in intracellular potassium content. The arabinitol produced during growth at a pH of 2.9 corresponded to 21 % of the energy content (kJ/l) of the total product formation or substrate consumption, or to 20 % of the glucose carbon consumed.

The effect of NaCl on growth of *S.cerevisiae* and *D.hansenii*

Both *S.cerevisiae* and *D.hansenii* changed the metabolic flow in response to increased external salinity. This was shown as a reduced growth yield, by 25 and 17 % in 0.68 and 1.35 M NaCl for *S.cerevisiae* and *D.hansenii*, respectively, (Table 2) and by an increased glycerol production, as shown for *D.hansenii* in Figure 5. For *D.hansenii* grown at 1.35 M NaCl, the glycerol production corresponded to 9 % of the energy content (kJ/l) of the total product formation or substrate consumption, as well as 8 % of the carbon content of the glucose consumed. The corresponding values for *S.cerevisiae* grown at 1.35 M NaCl, calculated from data given in ref. 12, was 38 and 32 %, respectively. The glycerol production constituted only a part of the additional energy expenditure which was used for growth at a high salinity (Fig. 6). When correcting the data for the energy change caused by the production of glycerol, the additional energy expenditure of *D.hansenii* for growth in 1.35 M NaCl as compared to biomass production in basic medium (0 M NaCl), was about -11 kJ per g of biomass formed, which corresponds to an additional energy expenditure of 65 %. When performing a maximal correction for the imbalance in the energy recovery, this additional energy expenditure corresponded to 39 %. The corresponding values for *S.cerevisiae* grown in 0.68 M NaCl were 49 and 33 %, respectively.

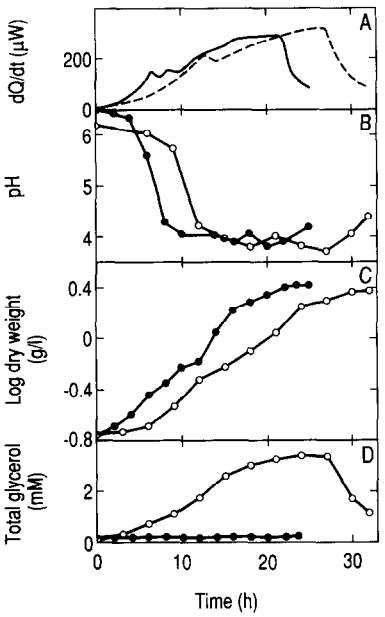
TABLE 2
Specific growth rates (μ) and growth yields ($Y_{X/S}$) during aerobic batch-growth of *D.hansenii* (*D.h.*) and *S.cerevisiae* (*S.c.*) at different external salinities and for *S.cerevisiae* also at different carbon sources.

NaCl (M)	Strain	Carbon Source	$Y_{X/S}$ (g/g)	$\mu_{R\text{-}F}$[a] (h^{-1})	μ_R[b] (h^{-1})
0	*S.c.*	Ethanol	0.397 ± 0.030 (SD, 9)		0.091±0.011 (SD, 9)
0	*S.c.*	Glucose	0.250 ± 0.018 (SD,20)	0.283±0.025 (SD,17)	0.032±0.007 (SD,16)
0.34	*S.c.*	Glucose	0.219 ± 0.013 (2)	0.246±0.09 (2)	0.033±0.001 (2)
0.68	*S.c.*	Glucose	0.187 ± 0.002 (2)	0.204±0.039 (2)	0.014±0.003 (2)
0	*D.h.*[c]	Glucose	0.480 ± 0.010 (SD, 3)		0.159±0.015 (SD, 4)
1.35	*D.h.*[c]	Glucose	0.400 ± 0.010 (2)		0.147±0.026 (SD, 3)

Variation as standard deviation or min-max deviation; number of experiments in brackets.
[a] Respiro-fermentative phase; [b] Respiratory phase; [c] Cultivation temperature 27°C.

Efficiency of growth of *S.cerevisiae* and *D.hansenii*

S.cerevisiae showed a low heat yield value of -9kJ/g, *i.e.* the amount of heat produced per unit of biomass formed during the respiro-fermentative growth on glucose, as compared to a heat yield value of -30 kJ/g for respiratory metabolism of glucose (Table 3). A low heat yield value may be interpreted as resulting from an efficient metabolism. However, most of the energy expenditure during the respiro-fermentative phase was excreted as ethanol during the respiro-fermentative metabolism (75 %), while the energy conserved as biomass amounted to only 14 % of the total product formation. For *D.hansenii* 60% of the energy was conserved as biomass, while 40 % was dissipated as heat (Table 3).



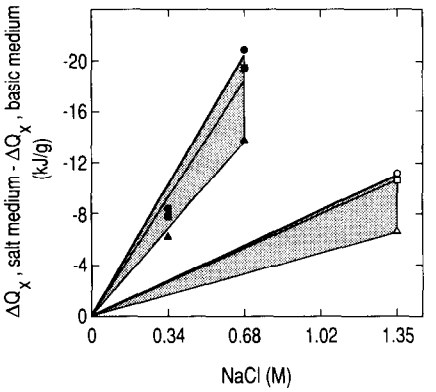


Fig. 5. (A) Rate of heat production, dQ/dt, (B) pH, (C) dry weight and (D) total glycerol production, expressed as mmoles per l of culture, during growth at 27°C of *D.hansenii* at a salinity of 0 M (full line and closed circles) and 1.35 M (broken line and open circles) NaCl, respectively. The data presented in this figure are re-drawn from data in ref. 12.

Fig. 6. Additional energy expenditure per unit of biomass produced (kJ/g) during growth of *S.cerevisiae* (closed symbols) and *D.hansenii* (open symbols; cultivation temperature 27°C) at different salinities. Original data (squares); data thermodynamically corrected for the glycerol production in salinity media (circles); data thermodynamically corrected for the glycerol production and for the imbalance in energy recovery (ER) (triangles).

TABLE 3
Heat yields (ΔQ_X), maximum specific growth rates (μ_{max}), and relative product formation calculated as the energy content of each product (kJ/l) in percentage of the total energy content of the products (kJ/l) during different metabolisms of *S.cerevisiae and D.hansenii*.

Strain	Metabolism	ΔQ_X	μ_{max}	Relative product formation[a] (%)				
		(kJ/g)	(h^{-1})	X	E	G	A	Q
S.cerevisiae	Respiro-fermentative[b]	- 8.6±0.8 (2)	0.3	14	75	4	1	6
S.cerevisiae	Respiratory[c]	-30.0±4.4 (SD,4)	-	40	-	-	-	60
D.hansenii	Respiratory[b]	-12.4±1.4 (SD,6)	0.3	60	-	-	-	40

[a] Biomass (X), ethanol (E), glycerol (G), acetate (A), heat (Q)
[b] Batch cultures
[c] Fed-batch cultures
Variation as standard deviation or min-max deviation; number of experiments within brackets.

DISCUSSION

Metabolic energy budgeting provides a tool in testing the consistency of the data in describing the reaction stoichiometry of the growth process at different physiological states (ref. 18, 19). For the experiments presented in this study, the average energy recoveries were 0.98 ± 0.02 (SD, 5exp.) and 1.06 ± 0.09 (SD, 12exp.) for the total growth process of *S.cerevisiae* and *D.hansenii*, respectively.

The calorimetrically measured heat dissipated during a metabolic process constitutes, in addition to being one of the components in the energy budget, also as a tool to study metabolic kinetics and to quantify the energy expenses of different metabolic processes during different environmental conditions. The evaluation of such quantifications has to be considered in the light of the experimentally obtained energy balance.

The metabolic rates of both *S.cerevisiae* and *D.hansenii* were strongly influenced by a change in pH (Figs. 1, 3). For *S.cerevisiae* the size-distribution of the catabolic flows in relation to biomass production did not seem to change significantly (Figs. 1C, 2) until a low pH of about 2.8 was reached, when a marked increase in heat yield was obtained (Fig. 1A, B) from about -47 kJ/g biomass formed to about -72 kJ/g (ref. 18). Such an increased energy expenditure for biomass production, may be interpreted as an increased maintenance requirement (ref. 20). For *D.hansenii*, the heat yield was already significantly increased at an external pH below 3.7 and at a pH of around 3 the energy expenditure for growth had more than doubled (Table 1), which means that the ATP requirement per unit of biomass formed more than doubled if an unchanged P/O-ratio is assumed. By adopting Pirt's equation (ref. 21), but using heat yields in the calculations, the maintenance requirements for growth at a pH around 3 was indicated to be doubled in comparison with growth at a pH around 3.5, *i.e.* values of -330 mW/g biomass and -170 mW/g biomass were attained, respectively. Growth stopped at a pH below 2.9. For an anaerobically grown *S.cerevisiae* (ref. 20), it was calculated that at a pH below 2.8 the fermentation rate limits the supply of ATP for maintenance purposes. A major role was ascribed to the pH effect on the plasma membrane ATPase and to an increased proton gradient across the plasma membrane, resulting in an increased passive proton uptake rate.

In addition to the increased energy expenditure (increased heat yield) of *D.hansenii* at a pH below 3.7, there was also a sharp increase in the production of arabinitol from a basic level of 0.7 mmoles/g biomass formed to 4 mmoles/g at a final pH of 2.9 (Fig. 4), where growth stopped. Arabinitol may act as a redox valve, as glycerol does for the anabolically produced NADH during anaerobic growth of *S.cerevisiae* (for review see ref. 22). For the growth period on urea at a pH of about 3 (Fig. 3), the arabinitol production corresponded to 95 % of the surplus of reducing equivalents formed during anabolism. In these calculations, the redox balance for the anabolic process of *S.cerevisiae* (ref. 22) was assumed to be valid also for *D.hansenii*. It thus seems as if the respiratory oxidation of cytoplasmatically produced reducing equivalents is limited at a low pH concerning *D.hansenii*. The arabinitol production during growth at a pH of about 3 corresponded to 18 % of the total energy content of the

substrates. If the energy expenditure caused by arabinitol production during growth at a pH of about 3 is added to the catabolic energy expenditure for growth, then the sum becomes about 2.5 times the corresponding energy expenditure at a pH of about 4.

The metabolic flow rates as well as the size-distribution of the metabolic flows were changed in response to increased external salinity during growth of *S.cerevisiae* and *D.hansenii* (Table 2, Fig. 5, ref. 12). If the indicated linear decrease of the growth yield with salinity for *S.cerevisiae* (Table 2) is assumed, then the decrease in growth yield is more than doubled at 1.35 M NaCl for *S.cerevisiae* (50 %) as compared to *D.hansenii* (17 %). A substantial part of the decrease in growth yield, *i.e.* about 41 and 15 % in 1.35 M NaCl for *S.cerevisiae* and *D.hansenii*, respectively, can be explained by the increase in glycerol production, for which the intracellular part is used for osmoregulation (ref. 5, 23). The additional decrease in growth yield is attributed to an increased catabolic energy expenditure for maintenance purposes in addition to the production of polyols during growth at increased salinities (Fig. 6). In 0.68 M NaCl, it corresponded to 33 to 49 % and 19 to 33 % of the basic energy expenditure for growth at 0 M NaCl for *S.cerevisiae* and *D.hansenii*, respectively, or in absolute values it was about 4 times larger for *S.cerevisiae* compared to that of *D.hansenii* (Fig. 6). Thus, it seems that *D.hansenii* is more efficient in coping with high salinities than *S.cerevisiae*, both concerning the production and maintenance related to the osmoregulation with glycerol and other maintenance requirements, *e.g.* for regulation of the intracellular ion composition (ref. 24-25). However, both because of indicated different efficiencies of the respiratory metabolism (Table 3) and of the mixed energy metabolism of *S.cerevisiae*, further studies are needed before a thorough evaluation can be made of the differences between the two yeasts. By using Pirt's equation (ref. 21), it can be calculated, however, that the increased maintenance requirements for *D.hansenii* during growth at pH 3 is of the same order as for growth at 1 M NaCl. This is in accordance with literature data for chemostat grown *S.cerevisiae* during the same pH and salinity conditions (ref. 20, 25).

In conclusion, in evaluating the efficiency of the response to differences in environmental pH and salinity of the two yeasts, both the effects on the maintenance requirement and the differences in polyol metabolism have to be considered. Taking both these aspects into account, the more energetically efficient *D.hansenii*, in terms of biomass yield (Table 2, 3), seems to be better adapted than *S.cerevisiae* to the often nutrient poor marine environment. *D.hansenii*, also seems to have adapted to the marine environment in that it showed a higher efficiency in coping with increased salinities, but showed a lower tolerance for a low pH than *S.cerevisiae*. In contrast, even though strain differences have to be taken into account (ref. 20), *S.cerevisiae* seems to be well adapted to nutrient rich environments, from where it has been isolated (ref. 1, 4), where a low pH may result from the metabolism of the microflora.

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