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Metabolic uncoupling in *Saccharomyces cerevisiae* ☆

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Abstract

Aerobic chemostat cultures of *Saccharomyces cerevisiae* were performed at different dilution rates under energy (glucose) limitation or at various extents of energy excess imposed by a nitrogen limitation.

It was found that energy excess induced metabolic uncoupling under steady-state conditions. The specific ethanol production rate was always higher the lower the feed medium nitrogen concentration throughout the whole range of dilution rates tested. In addition, the respiratory rate also increased under nitrogen limitation, for at least as long as the specific oxygen consumption rate was below the maximum capacity.

These results imply that the ATP yield (Y_{ATP} in g biomass per mol ATP) and/or the amount of ATP produced, i.e. the P/O ratio, must be able to change. By assuming a constant Y_{ATP} of 16 g mol^{-1}, a decrease in the P/O ratio from 1 to 1.5 at low dilution rates and glucose limitation down to only 0.1–0.2 at high dilution rates and nitrogen limitation could be calculated. If instead a fixed P/O ratio of 1.0 was assumed, the Y_{ATP} decreased from about 20 down to below 10 g mol^{-1} during these different conditions. Furthermore, the heat yield values, i.e. the amount of heat produced per amount of biomass formed, increased dramatically when the cells were subjected to a nitrogen limitation indicating less efficient growth in terms of conserving substrate energy as biomass under energy excess compared to energy limiting conditions.

Keywords: ATP; Biomass; Glycolysis; *Saccharomyces*; Uncoupling

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1. Introduction

The lack of coupling between anabolic requirements and catabolic energy production seems to be a common feature of many different microorganisms [1–4]. When there is an excess of energy source, the catabolic activity is often much higher than can be explained by anabolic ATP requirements. Consequently, this means that either the cells have to possess mechanisms for consuming ATP in processes not involving biomass formation or they must be able to produce less ATP for the same amount of substrate consumed compared to energy-limiting conditions. Of course, it may also involve a combination of these two possibilities.

The yeast *Saccharomyces cerevisiae* seems to be especially well adapted to metabolic uncoupling. When glucose-limited chemostat cultures of *Saccharomyces cerevisiae* and *Candida utilis* were pulsed with excess glucose, the two yeasts responded very differently [4]. *S. cerevisiae* increased its catabolic activity more or less immediately whereas the rate of biomass production remained unchanged for the first 30 min. *C. utilis*, however, immediately started to accumulate cell mass at a high rate [4]. These cells were adapted to an energy limitation when they were faced with a sudden energy excess. However, metabolic uncoupling has also been demonstrated for *S. cerevisiae* under steady-state conditions in chemostat cultures for cells adapted to energy excess [5].

In the study by Larsson et al. [5] metabolic uncoupling was induced by gradually decreasing the ammonium compared to the glucose concentration of the feed medium. During growth of *S. cerevisiae* with glucose as the sole limiting substrate, energy rather than carbon limits biomass formation [6,7]. Consequently, the cells shifted from energy limitation to energy excess when the supply of nitrogen was gradually decreasing. The aim of this study was to determine the resulting differences in total energy consumption, P/O ratio and ATP yield (amount of biomass formed per amount of ATP produced, Y_{ATP}) when the cells shifted from energy limitation to energy excess. The total energy consumption was measured as the amount of heat produced per amount of biomass formed, the so-called heat yield ($Y_{Q/X}$).

2. Experimental and calculations

The experimental conditions are described in Larsson et al. [5].

2.1. Yeast strain and media

S. cerevisiae CBS 426 was cultured in a medium with the following composition (per liter of solution): glucose, 20 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; NaCl, 0.5 g; yeast extract, 1 g; H_3BO_3, 1 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $MnCl_2$, 1 mg; $FeCl_3 \cdot 3H_2O$, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; Na_2MoO_4, 1 mg; KI, 0.1 mg; myoinositol, 25 mg; β-alanine, 2.5 mg; biotin, 7 mg; folic acid, 2 mg; *p*-aminobenzoic acid, 200 mg; nicotinic acid, 400 mg; pyridoxine hydrochloride,

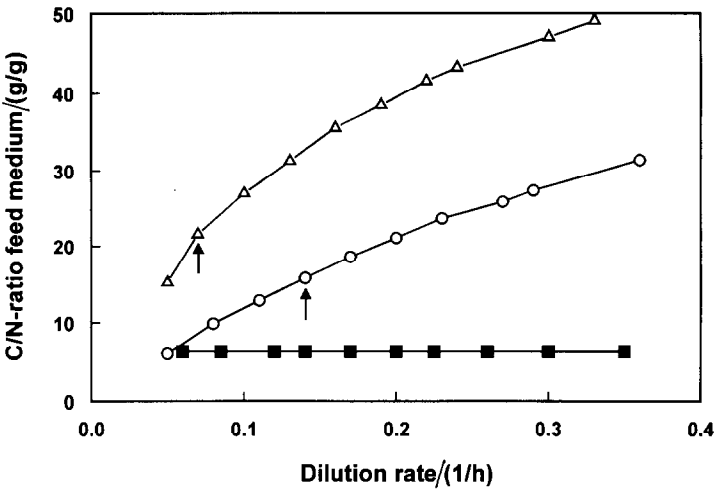

Fig. 1. Changes in the C/N ratio of the feed medium at different dilution rates for high (■), intermediate (○) and low (△) nitrogen concentration media. The arrows denote the start of nitrogen-limiting conditions. From Ref. [5], copyright © American Society of Microbiology.

400 mg; thiamine hydrochloride, 400 mg; riboflavine, 200 mg; Sigma Antifoam A, 0.2 ml.

The concentration of $(NH_4)_2SO_4$ was varied between the different experiments. In one experiment the $(NH_4)_2SO_4$ concentration in the feed was constant at 6 g l^{-1}. In the remaining experiments, two different medium reservoirs were used. The two reservoirs contained identical media except that one did not contain any $(NH_4)_2SO_4$. The flow of $(NH_4)_2SO_4$-containing medium was maintained constant, while the flow of the medium lacking $(NH_4)_2SO_4$ was varied in order to achieve the desired dilution rate. Consequently, increasing the dilution rate resulted in a decreasing feed concentration of $(NH_4)_2SO_4$. Furthermore, two different reservoir concentrations of $(NH_4)_2SO_4$ were employed. The resulting C/N ratios of the feed medium at different dilution rates are shown in Fig. 1. The arrows denote the onset of nitrogen limitation. This was determined as the point where the nitrogen content of the biomass started to deviate significantly compared to single glucose-limiting conditions. In the experiments with a constant C/N ratio, glucose was the sole limiting substrate throughout the whole range of dilution rates tested.

2.2. Growth conditions and calorimetry

The chemostat cultures were performed in a 2-l bench-scale calorimeter (model RC-1, Mettler-Toledo AG, Greifensee, Switzerland) modified for biological work. The working volume was 1.5 l, the temperature 30°C and the stirring rate was 300 rpm. Aeration was controlled at 1.5 vvm by a mass flow meter (5850 TR, Brookes Instrument, B.V., Veenendal, NL). The sterile filtered air (pore size 0.45 mm, Gelman Sciences Inc., Michigan, USA) was water-saturated by passing the air stream through a bubble column with a temperature of 33°C before entering the

chemostat. These conditions ensured a dissolved oxygen concentration above 60% of air saturation. The pH was kept constant at 4.5 by automatic addition of 4 M NaOH using a pH controller (Bioengineering AG, Wald, Switzerland). The feed medium was thermostated to 30°C before entering the chemostat.

The total activity (rate of heat production) of the culture was continuously monitored by the calorimeter. Before inoculation of the medium a base line for the calorimetric signal was established by running the calorimeter (chemostat) under identical conditions as the actual experiment for at least 12 h. The heat transfer coefficient (W m^{-2} K^{-1}) was then determined by measuring the response to the activation of a 10 W in situ calibration heater for 20 min. The principles of the calorimetric measurement procedure have been described previously [8].

After inoculation, substrate feeds for continuous operation were initiated once the cells had reached the late exponential or stationary phase of batch growth. The experiments were always performed by starting at the lowest dilution rate and subsequently increasing the dilution rate step by step. Apart from continuous on-line measurement of the rate of heat production by the cells, samples were taken, once a steady state had been established, for determination of biomass concentration, extracellular concentrations of glucose, ethanol, acetate, glycerol and ammonium and for determination of the elemental composition of the biomass.

2.3. Test for steady-state conditions

A constant heat production rate, as recorded by the calorimeter, was used as the criterion for the establishment of steady-state conditions. All reactions in a cell give rise to a heat change; therefore, the calorimetric technique can be used as a measure of the total activity of the culture [9–11].

2.4. Dry weight determinations

Samples (2 × 4 ml) were centrifuged for 5 min at 3500 *g*, washed twice with deionized water, dried for 24 h at 105°C and stored in a desiccator before weighing.

2.5. Determinations of glucose, ethanol, acetate, glycerol and ammonium concentrations

Samples (2 × 4 ml) were centrifuged for 5 min at 3500 *g*. The supernatants were stored in the freezer (−20°C). Glucose, ethanol, acetate, glycerol and ammonium were determined using enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Mannheim, Germany).

2.6. Gas analysis

The concentrations of CO_2 and ethanol in the exhaust gas stream from the reactor were determined by infrared spectroscopy (model PSA-401, Servomex, Crowborough, UK). The sample line from the reactor and the gas distribution system were heated in order to prevent condensation of ethanol in the gas stream.

2.7. Calculation of oxygen consumption

Calculation of the specific oxygen consumption rate (dO_{2sp}/dt in mol g^{-1} h^{-1}) was performed according to Larsson et al. [5]. The measured total rate of CO_2 produced, minus the CO_2 production which accompanies the formation of ethanol and biomass minus 0.5 times the rate of glycerol produced, divided by the actual biomass concentration equals the specific oxygen consumption rate.

2.8. Calculation of heat yield

Calculation of the heat yield ($Y_{Q/X}$ in kJ g^{-1}) was done by dividing the steady-state rates of heat production (kJ l^{-1} h^{-1}) by the steady-state rate of ash-free biomass formation (g l^{-1} h^{-1}).

2.9. Calculation of ATP yield

The ATP yield (Y_{ATP} in g biomass formed per mol ATP produced) was calculated according to

$$Y_{ATP} = 1/\{[Y_{ATP/O_2}(dO_{2sp}/dt)] + [Y_{ATP/EtOH}(dEtOH_{sp}/dt)] + [Y_{ATP/Ac}(dAc_{sp}/dt)] - [Y_{ATP/Gly}(dGly_{sp}/dt)]\}D \quad (1)$$

where Y_{ATP/O_2} is the amount of ATP formed per amount of oxygen consumed (mol ATP per mol O_2), dO_{2sp}/dt is the specific oxygen consumption rate (mol g^{-1} h^{-1}), $Y_{ATP/EtOH}$ is the amount of ATP formed per amount of ethanol produced (mol ATP per mol ethanol), $dEtOH_{sp}/dt$ is the specific ethanol production rate (mol g^{-1} h^{-1}), $Y_{ATP/Ac}$ is the amount of ATP formed per amount of acetate produced (mol ATP per mol acetate), dAc_{sp}/dt is the specific acetate production rate (mol g^{-1} h^{-1}), $Y_{ATP/Gly}$ is the amount of ATP consumed per amount of glycerol formed (mol ATP per mol glycerol), $dGly_{sp}/dt$ is the specific glycerol production rate (mol g^{-1} h^{-1}), and D is the dilution rate (h^{-1}).

The value of Y_{ATP/O_2} depends on the P/O ratio. The values used were 4.67 mol ATP per mol O_2 at P/O = 2, 2.67 at P/O = 1, and 1.67 O_2 at P/O = 0.5. The production of NADH and FADH yields the same amount of ATP because, at least during growth, the electron transport chain of *S. cerevisiae* lacks the first proton translocating site [12]. Note that these values of Y_{ATP/O_2}, not only include electron transport phosphorylation, but also substrate level phosphorylation in glycolysis and the TCA cycle. In addition, the NADH produced during acetate and biomass [13] formation and NADH consumption during glycerol formation are already compensated for in the calculation of specific oxygen consumption rate. Formation of 1 mol ethanol or acetate yields 1 mol ATP produced whereas 1 mol glycerol produced consumes 1 mol of ATP.

2.10. Calculation of the P/O ratio

The P/O ratios were calculated by assuming a constant ATP yield of 16 g biomass per mol ATP and by Eqs. (2) and (3)

$$\{[Y_{ATP/O_2}(dO_{2sp}/dt)] + [Y_{ATP/EtOH}(dEtOH_{sp}/dt)] + [Y_{ATP/Ac}(dAc_{sp}/dt)] - [Y_{ATP/Gly}(dGly_{sp}/dt)]\}/D = 1/Y_{ATP} \quad (2)$$

and

$$P/O = (Y_{ATP/O_2} - Y_{ATP/O_2}S)/2 \quad (3)$$

where $Y_{ATP/O_2}S$ is the ATP production from substrate level phosphorylation in glycolysis and TCA cycle (mol ATP per mol O_2). The value used in the calculations was 2/3 or 0.67.

The value of 16 g biomass per mol ATP has been determined in anaerobic glucose-limited chemostat cultures of *S. cerevisiae* at $D = 0.10\ h^{-1}$ under optimal growth conditions by Verduyn et al. [14].

2.11. Energy balance

The average values of the energy balance, including all the energy content of the yeast extract of 16.67 kJ g^{-1}, for the different dilution rates, were 0.92 ± 0.05 (SD, $n = 10$), 0.99 ± 0.07 (SD, $n = 10$), and 0.99 ± 0.06 (SD, $n = 10$) for the high, intermediate and low nitrogen experiments, respectively. Neglecting the contribution from the yeast extract yielded an average energy balance of 0.97 ± 0.05, 1.04 ± 0.7 and 1.06 ± 0.06 for high, intermediate and low nitrogen experiments.

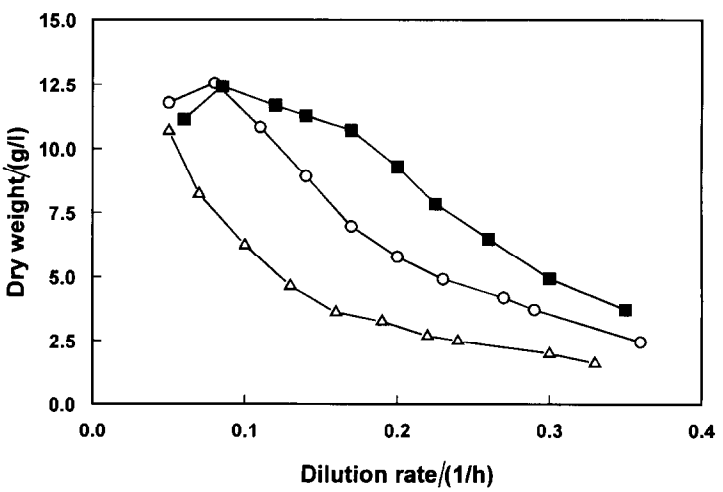

Fig. 2. Changes in dry weight at different dilution rates during aerobic growth of *S. cerevisiae* with 20 g l^{-1} of glucose in high (■), intermediate (○) and low (△) nitrogen concentration media.

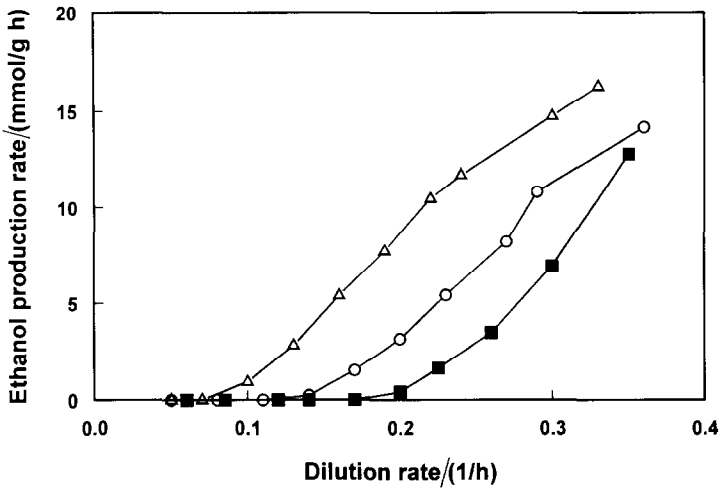

Fig. 3. Changes in specific ethanol production rate at different dilution rates during aerobic growth of *S. cerevisiae* in high (■), intermediate (○) and low (△) nitrogen concentration media.

3. Results and discussion

Aerobic chemostat cultures were performed at different dilution rates under energy (glucose) limitation or at various extents of energy excess imposed by a nitrogen limitation.

3.1. Biomass concentration

The biomass concentration decreased when the nitrogen content of the feed medium was reduced (Figs. 1 and 2). Due to the limited respiratory activity of *S. cerevisiae* [15,16] not only respiration but also fermentation is used at higher dilution rates. Because fermentation results in a much lower yield compared to respiration, the biomass concentration diminished at higher dilution rates also under glucose limitation alone (Fig. 2).

3.2. Ethanol production

The onset of fermentation was found at a much lower dilution rate under nitrogen limitation, with a value of 0.10 h^{-1}, compared to 0.20 h^{-1} under glucose limitation (Fig. 3). In addition, the specific ethanol production rate was, at each dilution rate, always higher the lower the medium nitrogen concentration (Fig. 3). Substituting respiration with fermentation might be a way of producing less ATP under energy excess when the anabolic requirements are lower due to the limited supply of nitrogen, unless, of course, the respiratory activity remains the same under energy limitation and energy excess and the increased fermentation activity is added to it.

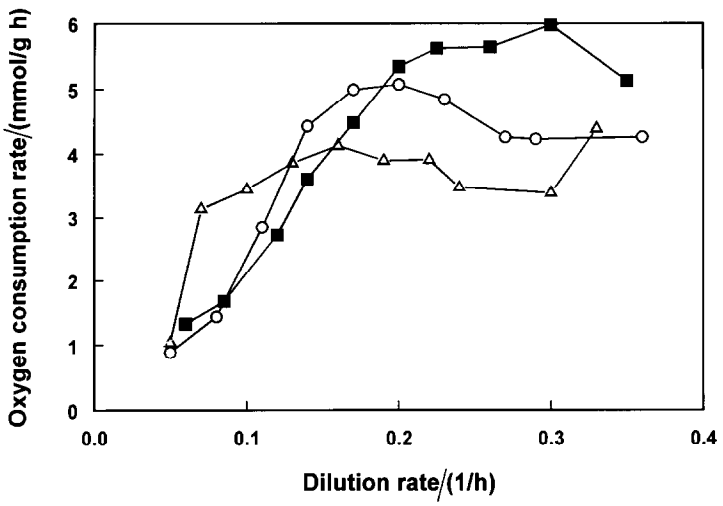

Fig. 4. Changes in specific oxygen consumption rate at different dilution rates during aerobic growth of *S. cerevisiae* in high (■), intermediate (□) and low (△) nitrogen concentration media.

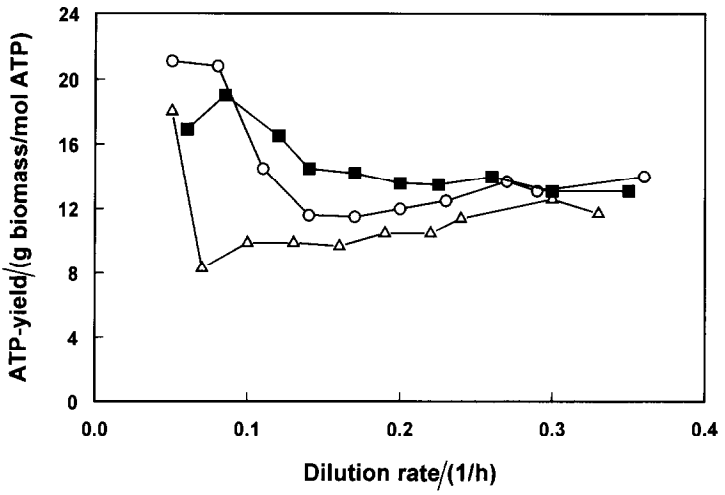

Fig. 5. Changes in ATP yield at different dilution rates during aerobic growth of *S. cerevisiae* in high (■), intermediate (○) and low (△) nitrogen concentration media. The calculations were made by assuming a constant P/O ratio of 1.0.

3.3. Oxygen consumption

Surprisingly enough, the specific oxygen consumption rate responded in a similar fashion as the specific ethanol production rate, at least at low dilution rates. In other words, the respiratory activity was also higher at lower medium nitrogen concentrations (Fig. 4). For instance, the oxygen consumption rate at $D = 0.07\ h^{-1}$ under nitrogen limitation was more than doubled compared to similar dilution rates

under glucose limitation. The difference compared to the specific ethanol production rate was that the cells reached a saturation level of the respiratory activity. Furthermore, this saturation level decreased with decreasing medium nitrogen concentration (Fig. 4).

3.4. ATP yield

The ATP yield was calculated by assuming a constant P/O ratio of 1.0 [6,17]. Even under single glucose limitation, the Y_{ATP} decreased from about 20 at low dilution rates down to 13 g biomass per mol ATP at higher values of D (Fig. 5). A decrease in Y_{ATP} could be expected because *S. cerevisiae* contains an increasing amount of protein at higher dilution rates [6,14,18]. Nitrogen limitation and energy excess caused a very rapid reduction of the Y_{ATP}, especially at low dilution rates (Fig. 5). If the cells had started to produce large amounts of glycogen or trehalose at low dilution rates and nitrogen limitation this would have the opposite effect. In terms of gram substance per mol ATP it is much cheaper to produce glycogen or trehalose compared to a complete cell, i.e. the ATP yield ought to increase under such conditions. The difference in Y_{ATP} between glucose limited (energy limitation) and nitrogen limitation (energy excess) was almost completely lost at the highest dilution rates tested (Fig. 5).

Perhaps a "wasteful" energy utilization is a prerequisite for obtaining a high growth rate. Alternatively, high growth rates may require a certain protein content of the cells. Because protein synthesis is one of the most energy-demanding processes in the synthesis of a cell [19], the ATP yield might simply be determined by the high rate of protein synthesis of these high dilution rates. However, it may also be that the P/O ratio decreased, i.e. less ATP was produced when the anabolic demands were diminishing due to a limited supply of nitrogen.

3.5. P/O ratio

In order to calculate P/O ratios, a constant Y_{ATP} of 16 g biomass per mol ATP was assumed. This is a value which has been determined for *S. cerevisiae* in anaerobic chemostat cultures under optimal growth conditions and with glucose as the limiting substrate [14]. The P/O ratio was about 1–1.5 at low dilution rates and glucose limitation (Fig. 6). When the cells were subjected to a nitrogen limitation and energy was present in excess the ratio was drastically reduced. The values were as low as 0.1–0.2 at high dilution rates and nitrogen limitation. Also the growth rate seemed to influence the P/O ratio. There was a more or less continuous decrease in the P/O ratio with increasing dilution rate even for the single glucose-limited culture (Fig. 6). Of course, this may be a combined effect of a decreasing P/O ratio and of a lower ATP yield at high dilution rates due to the increasing protein content of the cells as discussed above.

3.6. Heat yield

The heat yield, i.e. the amount of heat produced per amount of biomass formed, is independent of biomass concentration as well as growth rate. Consequently, high

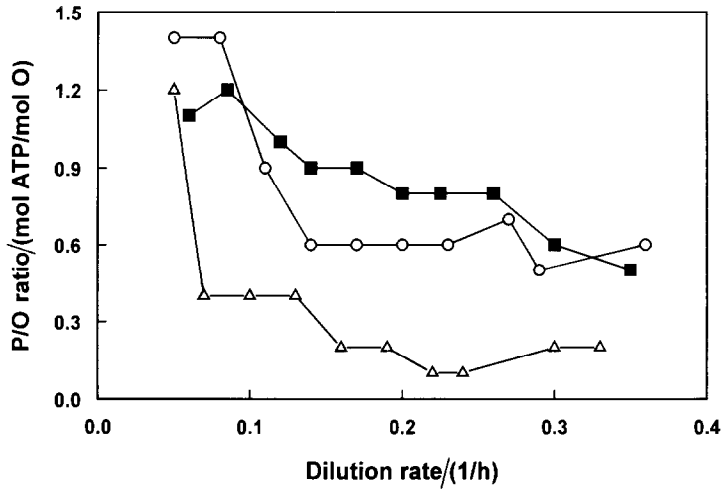

Fig. 6. Changes in P/O ratio at different dilution rates during aerobic growth of *S. cerevisiae* in high (■), intermediate (○) and low (△) nitrogen concentration media. The calculations were made by assuming a constant ATP yield of 16 g biomass per mol ATP.

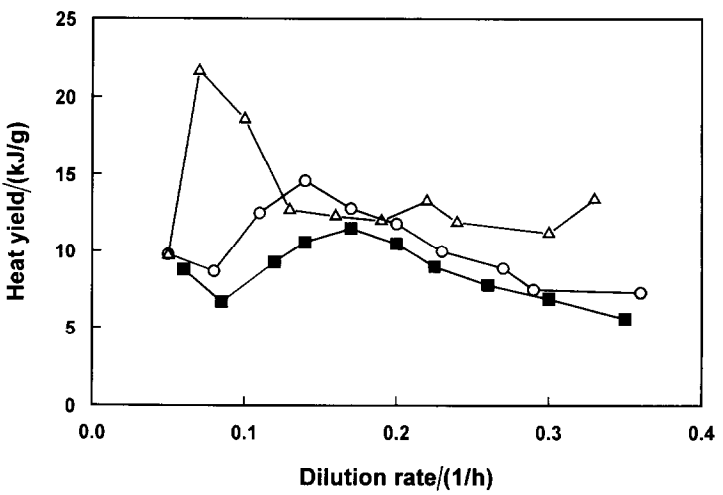

Fig. 7. Changes in heat yield at different dilution rates during aerobic growth of *S. cerevisiae* in high (■), intermediate (○) and low (△) nitrogen concentration media. From Ref. [5], copyright © American Society for Microbiology.

heat yield values indicate inefficient growth if the comparison is made with cells using the same metabolism. Here, the word inefficient is used in the sense of conserving substrate energy as biomass. As expected, the heat yield values did increase under nitrogen (energy excess) compared to glucose (energy limitation) limitation (Fig. 7). At higher dilution rates the heat yield values decreased. This is simply because at higher dilution rates the cells used fermentation in addition to

respiration. Fermentation of glucose yields only 100 kJ per mole glucose, whereas respiration yields 2814 kJ per mol glucose [20]. However, the heat yield values were always higher under nitrogen compared to glucose limitation, also at higher dilution rates (Fig. 7), even though fermentation was even more dominating in these cells (Figs. 3 and 4). This clearly indicates that either the ATP yield and/or the P/O ratio changed under nitrogen limitation.

4. Conclusions

To summarize, not only the fermentative but also the respiratory activity of the cells increased when the conditions changed from energy limitation to energy excess (Figs. 3 and 4). This means that the cells have either to consume ATP in processes not coupled to growth, i.e. the ATP yield will decrease, or that mechanisms exist which result in less ATP produced for the same amount of substrate consumed. The ATP produced via respiration might be regulated in order to meet the anabolic demands by a changing P/O ratio. Under fermentative conditions, however, there is a strict coupling between the amount of products formed and ATP produced by substrate phosphorylation unless futile cycles, such as between fructose 1,6-phosphate and fructose 6-phosphate, exists in glycolysis. If this is the case there would be no longer any strict coupling between product formation and ATP production. Another way of producing less ATP for the same amount of substrate consumed under fermentative conditions would be to use alternative pathways. For instance, some microorganisms have the capability of using the methylglyoxal bypass, thereby circumventing the ATP-producing steps of glycolysis [1]. In *S. cerevisiae*, glycerol production would give the same effect, i.e. circumventing the energy-generating steps of glycolysis. However, our results cannot be explained by an increased glycerol production because its specific production rate was more or less the same irrespective of glucose or nitrogen limitation (data not shown). Similar results have also been reported for anaerobic chemostat cultures of *S. cerevisiae* where uncoupling was induced by limiting the supply of nitrogen, i.e. the glycerol yield was not higher during nitrogen compared to glucose limitation [21]. If ATP is produced in excess of the minimum requirements for biomass production, this excess may be consumed by, for example, pumping of protons or any other ion due to an increased permeability through the membrane. It is known that weak acid, because of the resulting extra energy requirement for proton pumping, induces uncoupling and a decreased ATP yield in *S. cerevisiae* [14]. The results concerning acetate concentrations in this study did not, however, correlate with uncoupling (data not shown). It may also be that the excess ATP is consumed by an increased turn-over rate of proteins or any other cell component.

Similarly, this would also result in a reduced ATP yield. It should be noted that, if futile cycles in glycolysis exist, these will also result in an apparent decrease in the ATP yield. In this case, however, it is the amount of ATP produced that has changed rather than the ATP yield.

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