Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures

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The energetics of *Saccharomyces cerevisiae* were studied in anaerobic glucose-limited chemostat cultures via an analysis of biomass and metabolite production. The observed Y_{ATP} was dependent on the composition of the biomass, the production of acetate, the extracellular pH, and the provision of an adequate amount of fatty acid in the medium. Under optimal growth conditions, the Y_{ATP} was approximately 16 g biomass (mol ATP formed)$^{-1}$. This is much higher than previously reported for batch cultures. Addition of acetic acid or propionic acid lowered the Y_{ATP}. A linear correlation was found between the energy required to compensate for import of protons and the amount of acid added. This energy requirement may be regarded as a maintenance energy, since it was independent of the dilution rate at a given acid concentration.

Introduction

It has become widely accepted that the anaerobic Y_{ATP} of yeasts is approximately 10·5 g cells (mol ATP formed)$^{-1}$, as originally reported in the classical study of Bauchop & Elsden (1960). Nevertheless, this value contains an important flaw, since no correction has been made for the obligatory, energy-requiring production of glycerol, which acts as a redox sink under anaerobic conditions (Oura, 1977). Furthermore, most values of Y_{ATP} obtained for yeasts have been for batch cultures. In this respect Haukeli & Lie (1971) reported a sharp decline in the Y_{ATP} of various yeasts, grown anaerobically, as a function of the number of generations, with values decreasing from a maximum of 17 at the early stages of growth, to a value of approximately 11 g cells (mol ATP formed)$^{-1}$ at later stages.

Dekkers *et al.* (1981) calculated a Y_{ATP} of 8·6 (after correction for glycerol formation) for anaerobic growth of *Saccharomyces cerevisiae* CBS 426 in chemostat culture. From the data of Schatzmann (1975), an anaerobic Y_{ATP} (at $D = 0·10$ h^{-1}) of approximately 16·5 can be calculated for *S. cerevisiae* H1022 in continuous culture, much higher than the previous estimates from batch cultures, and twice as high as the value obtained by Dekkers *et al.* (1981).

In view of these contradictory results, we studied the energetics of two *S. cerevisiae* strains during anaerobic chemostat cultivation. In this paper evidence is provided

that the anaerobic Y_{ATP} is much higher than previously estimated with batch cultures. Y_{ATP} is influenced by variations in cell composition, excretion of weak acids, and by the amount of unsaturated fatty acid in the growth medium.

Methods

Micro-organisms and cultivation. The methods of anaerobic cultivation of *S. cerevisiae* CBS 8066 and *S. cerevisiae* H1022, and of analysis of metabolites are described in the accompanying article (Verduyn *et al.*, 1990).

Calculation of Y_{ATP}. Under anaerobic conditions the Y_{ATP} can be calculated by dividing the biomass concentration by the difference of the concentrations of metabolites formed with net production of ATP (mainly ethanol), and of those requiring a net input of ATP (mainly glycerol). Thus

$$Y_{ATP} = \frac{[\text{biomass}]}{[\text{ethanol}] - [\text{glycerol}]} \quad (1)$$

where Y_{ATP} is expressed as g cells formed per mol of ATP produced.

Although the production of some of the minor byproducts, such as acetate or pyruvate, also yields energy, their concentrations usually are so low that they hardly influence the observed Y_{ATP} directly. Therefore they have been neglected in our calculations. Provided that the maintenance requirement [m_e; mmol ATP (g biomass)$^{-1}$ h^{-1}] is known, the Y_{ATP} corrected for maintenance, the so-called Y_{ATP}^{max} (Stouthamer, 1973; Stouthamer & Bettenhaussen, 1973) can be calculated from the equation

$$\frac{1}{Y_{ATP}} = \frac{m_e}{\mu} + \frac{1}{Y_{ATP}^{max}} \quad (2)$$

In calculating m_e it is assumed that glucose needed for maintenance is completely converted into ethanol with an ATP yield of 2 mol (mol glucose)$^{-1}$.

A theoretical value for Y_{ATP} can be obtained via an estimation of the amount of energy required for production of a given amount of biomass, by a theoretical analysis of all the assimilatory reactions leading to the formation of biomass. This requires knowledge of the cell composition, both in terms of biomass polymers (proteins, carbohydrates, RNA and lipids) and of the monomer composition (amino acids, nucleotides etc.) of these polymers. From known biochemical routes, it is then possible to calculate the minimal amount of ATP required for the biosynthesis of the various cell components, leading to a maximal value for the Y_{ATP}^{max}, which is indicated here as the theoretical Y_{ATP}^{max} (Stouthamer, 1973).

Calculation of the theoretical Y_{ATP}^{max}

Composition and synthesis of polymers. The total protein content of the yeast was determined experimentally (Verduyn *et al.*, 1990). The RNA content was taken from a survey of literature data, indicating that the average RNA content in yeasts during chemostat cultivation ($D = 0.10$ h^{-1}) is $7 \pm 3\%$ (Alroy & Tannenbaum, 1973; Chistyakova *et al.*, 1983; Furukawa *et al.*, 1983; Parada & Acevedo, 1983). However, the RNA content of micro-organisms increases with increasing dilution rates (Furukawa *et al.*, 1983; Parada & Acevedo, 1983).

The fatty-acid content during anaerobic growth was calculated from the amount of fatty acid present in the medium, assuming that this was completely incorporated into biomass. This gave a value for lipid content of approximately 3.5% of the dry weight (Verduyn *et al.*, 1990). All carbohydrate was taken as polyglucose with a molecular mass of 162 Da and an energy requirement for synthesis of 2 mmol ATP (mmol polymer)$^{-1}$. It was assumed that the polymers, together with phosphorus and sulphur, make up 95% of the dry weight of biomass; consequently, the carbohydrate content can be calculated as $95\% - \%$ protein $- 10.5\%$(RNA + lipid content). This slightly overestimates the carbohydrate content, since phosphorus is not always directly incorporated into biomass (nucleic acids and phospholipids), but is also found as inorganic phosphate and polyphosphate. Total phosphorus and sulphur were assumed to be 2.9% and 2% of the dry weight, respectively. Of the various metal ions found in the ash, potassium is by far the largest (in weight-percentage), namely 2.2% of the total dry weight (Conway & Armstrong, 1961; Aiking & Tempest, 1976; Maiorella *et al.*, 1984).

Composition, synthesis and polymerization of monomers. For the amino acid composition, the average values determined by Oura (1972) for *S. cerevisiae* were used, but were adjusted to the experimentally determined protein contents of the yeasts which were higher than the value used by Oura. Pathways of amino acid biosynthesis were taken from Umbarger (1978). Compared to the calculations of Oura some changes were introduced. For instance, during the synthesis of amino acids net production of xylulose 5-phosphate occurs. Oura considered this to be reconverted into glucose. It can, however, be used directly as a precursor in the synthesis of nucleotides. It was also accepted that polymerization of amino acids requires 4 mol ATP (mol amino acid)$^{-1}$ (Stanier *et al.*, 1987) rather than 2 mol.

In biosynthetic reactions, it was inferred that oxaloacetate is formed from pyruvate by pyruvate carboxylase; formation of acetyl-CoA was assumed to occur via pyruvate dehydrogenase.

The biosynthesis of RNA was adopted from Stanier *et al.* (1987). Since the DNA content of yeasts is only $0.2-0.3\%$ of the dry weight (Oura, 1972; Furukawa *et al.*, 1983), all nucleic acids were regarded as RNA. The composition of RNA was taken from Bruinenberg *et al.* (1983). For the calculation of the turnover of mRNA, the values of Stouthamer (1973) were used, with a correction for the lower RNA content of yeasts as compared to bacteria.

The generation of NADPH via the hexose monophosphate pathway also requires energy (Bruinenberg *et al.*, 1983). For simplicity, it was assumed that glucose is completely oxidized to CO_2 via the hexose monophosphate route, with a yield of 12 NADPH per glucose (Bruinenberg *et al.*, 1983). Hence the synthesis of 1 mol NADPH requires 1/12 mol ATP, due to the phosphorylation of glucose to glucose 6-phosphate.

Energy required for transport. Most substances taken up by yeasts, including ammonium, phosphate and potassium ions, are actively transported across the plasma membrane (for a review see Eddy, 1982). A notable exception is glucose which, in *S. cerevisiae*, is transported by facilitated diffusion (Romano, 1982; Lang & Cirillo, 1987). In order to calculate the transport costs, the H$^+$/ATP-stoichiometry of the plasma membrane ATPase must be known. There is now a general consensus that this value is 1, as determined for enzyme preparations of a number of eukaryotes, including *S. cerevisiae* (Malpartida & Serrano, 1981), the fungus *Neurospora crassa* (Perlin *et al.*, 1986) and mammals (Nelson & Taiz, 1989). A value of 1 can also be calculated from data for *S. cerevisiae* in Dufour *et al.* (1982). It should be noted that active uptake of various nutrients is driven by the protonmotive force, and not directly by ATP itself. Therefore, transport costs are expressed as 'ATP-equivalents' (ATP$_{eq}$). In the theoretical calculation of the transport costs, it was assumed that transport of ammonium and potassium ions requires 1 ATP$_{eq}$ and that of phosphate 2 ATP$_{eq}$. The N-requirement in the form of ammonium was calculated from the experimentally determined N-content.

Results

Y_{ATP} as a function of growth rate and medium composition

Y_{ATP} as calculated from biomass and metabolite concentrations decreased when the dilution rate was increased from 0.05 to 0.28 h^{-1} (Table 1, Fig. 1). The protein content of the biomass increased from 47 to $57 \pm 2\%$ (Fig. 1). A corresponding increase was found in the N-content. Also, a progressive increase in the extracellular acetate concentration (0.4 to 0.9 mM) in cultures of *S. cerevisiae* CBS 8066 was observed (Fig. 1).

At a dilution rate of 0.10 h^{-1}, *S. cerevisiae* CBS 8066 had a Y_{ATP} of 14.0, whereas a value of 15.8 g cells (mol ATP)$^{-1}$ was calculated for *S. cerevisiae* H1022 (Table 1). These values apply, of course, only to optimal culture conditions. For example, when the pH was decreased, a drastic reduction in Y_{ATP} values was observed (see Table 4). Furthermore, the Y_{ATP} was also critically dependent on other parameters such as the amount of oleic acid in the growth medium. Unsaturated fatty acids are thought to be essential for anaerobic growth of *S. cerevisiae* (Andreasen & Stier, 1953, 1954). As reported in the accompanying paper (Verduyn *et al.*, 1990) suboptimal concentrations of unsaturated fatty acid lead to uncoupling of growth and energy production. In the absence of Tween 80 as a source of oleic acid, the Y_{ATP} of *S. cerevisiae* H1022 was as low as 9.5 g cells (mol ATP)$^{-1}$ (data not shown).
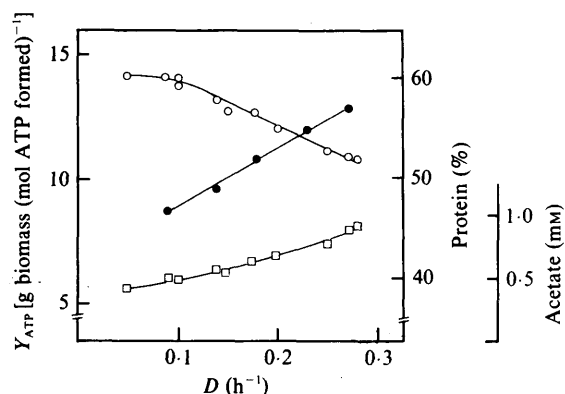
Fig. 1. Y_{ATP} (O), protein content (●) and acetate excretion (□) as a function of the dilution rate during anaerobic glucose-limited chemostat cultivation of *S. cerevisiae* CBS 8066 (pH 5·0).

Table 1. *Anaerobic growth of S. cerevisiae CBS 8066 and S. cerevisiae H1022 in glucose-limited chemostat cultures as a function of the dilution rate*

Growth was at pH 5·0; the glucose concentration in the reservoir was 23 g l⁻¹. Maximal variations in the data (as a percentage of the absolute values) were: 2% for biomass and C-content; 3% for ethanol; 4% for protein and N-content; 5% for glycerol; and 20% for acetate, succinate, pyruvate and residual glucose. ND, Not determined.

	Dilution rate (h⁻¹):		
	0·10	0·20	0·27
Strain ...	1022 8066	8066	8066
Glucose (g l⁻¹)	0·05 0·07	0·19	0·65
Biomass (g l⁻¹)	2·64 2·36	2·14	1·93
Cell yield (g g⁻¹)	0·115 0·103	0·094	0·086
Protein content (%)	45 47	53	57
C-content (%)	ND 45·0	45·4	45·3
N-content (%)	ND 8·8	9·9	10·9
Ethanol (mM)	191 190	200	198
Glycerol (mM)	23·6 21·0	22·6	22·1
Acetate (mM)	0·04 0·5	0·7	0·9
Pyruvate (mM)	0·15 0·2	0·4	0·6
Succinate (mM)	1·1 0·9	1·5	1·8
Carbon recovery (%)	98 96	99	98
Y_{ATP} [g (mol ATP)⁻¹]	15·8 14·0	12·1	11·0

Theoretical analysis of the relationship between biomass composition and energetics

A theoretical analysis of the energy requirements for the formation of the various cell constituents shows that the polymerization of amino acids is the main ATP-consuming process in the formation of yeast biomass (Table 2). Small variations in the contents of RNA, lipid or carbohydrate will not significantly affect the theoretical Y_{ATP}^{max}. It can therefore be expected that the increase in protein content with increasing dilution rate (Fig. 1) will

Table 2. *Calculation of the theoretical Y_{ATP}^{max} for anaerobic glucose-limited chemostat growth of S. cerevisiae CBS 8066*

Growth was at pH 5·0 and $D = 0·10$ h⁻¹. The cell composition was taken as 47% protein, 37·5% carbohydrate, 7% RNA, 3·5% fatty acid and 5% ash; see also Methods.

	ATP requirement [mmol ATP (100 g biomass)⁻¹]
Amino acid synthesis	181
Amino acid polymerization	1776
Carbohydrate synthesis	463
Fatty acid synthesis	0*
RNA synthesis and polymerization	135
Turnover of mRNA	71
NADPH synthesis	44
Transport of ions	
Ammonium	629
Potassium ⎫ Phosphate ⎭	240
Total	3539

Theoretical Y_{ATP}^{max} ... 28·3 g biomass (mol ATP)⁻¹

* Assumed to be taken up from the medium and incorporated into biomass in the form of oleic acid.

result in a reduction of Y_{ATP}. From the data presented in Table 2 it can be calculated that the theoretical Y_{ATP}^{max} should decrease from 28·3 at $D = 0·1$ h⁻¹ to 25·0 at $D = 0·28$ h⁻¹ (the RNA content is assumed to increase from 7 to 10%; Furakawa *et al.*, 1983). This corresponds to an increase in ATP requirement of 5 mmol (g cells)⁻¹. Experimentally, a decrease in Y_{ATP} from 14·0 to 11 was observed for *S. cerevisiae* CBS 8066 (Fig. 1), corresponding to a difference of 20 mmol ATP (g cells)⁻¹. From these data it is apparent that a large discrepancy exists between the theoretical and the experimental Y_{ATP}. This fact has been recognized for many years and has also been observed for bacteria; it remains one of the outstanding problems in the bioenergetics of micro-organisms. Factors contributing to this gap might include futile cycles, membrane permeability to protons etc. (Lagunas, 1976; Stouthamer, 1979; Tempest & Neijssel, 1984).

The decrease in the experimentally determined Y_{ATP} with increasing dilution rates is much larger than expected from theoretical calculations. However, the decrease in biomass yield may be due not only to an increased energy requirement as a consequence of changes in biomass composition. It may also be caused by weak acids, which are known to have an uncoupling effect (Baronofsky *et al.*, 1984; Alexander *et al.*, 1987; Postma *et al.*, 1989). These acids progressively increased in concentration with increasing dilution rates (Table 1, Fig. 1).

The fact that acetate formation was observed under all experimental conditions in anaerobic cultures of *S. cerevisiae* CBS 8066 complicates an estimation of the 'real' Y_{ATP} (i.e. without uncoupling by acetic acid). Therefore, some experiments were done with *S. cerevisiae* H1022, since it has been reported that this particular strain does not excrete acetate (Schatzmann, 1975). This was confirmed in our study (Table 1). Indeed, Y_{ATP} calculated on the basis of metabolites formed at a dilution rate of 0·10 h^{-1} and pH 5·0 was higher for H1022 than for CBS 8066: 15·8 versus 14·0 g biomass (mol ATP formed)$^{-1}$ (Table 1). From the data for H1022 given by Schatzmann (1975), a value for Y_{ATP} of 16·5 can be calculated for a dilution rate of 0·10 h^{-1}.

Effect of weak acids on the energetics of glucose-limited chemostat cultures

Addition of propionate to the medium reservoir results in a severe reduction of the biomass yield of both yeast strains, with a concomitant increase in specific ethanol production (Verduyn *et al.*, 1990). Therefore, a progressive decrease of the Y_{ATP} will occur upon addition of increasing concentrations of propionate. Representative data on the effect of propionate on *S. cerevisiae* H1022 are summarized in Table 3. Since with both strains washout occurred at a concentration of 25 to 30 mM added propionate, it can be concluded that both yeasts are equally susceptible to the uncoupling effect of weak acids. Consequently, it is likely that the 'true' Y_{ATP} for *S. cerevisiae* CBS 8066 is approximately the same as that for strain H1022, i.e. 16 g biomass (mol ATP formed)$^{-1}$.
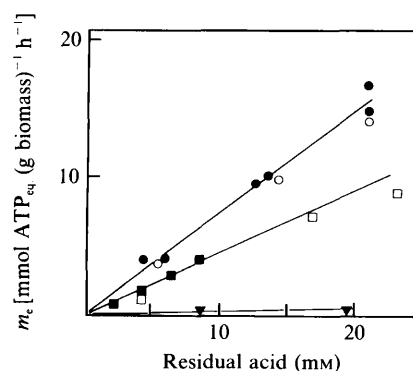

Fig. 2. Calculated m_e as a function of the residual acid concentration in the culture following addition of acetate ($\square$, $\blacksquare$), propionate ($\bigcirc$, $\bullet$) or succinate ($\triangledown$, $\blacktriangledown$) to the medium reservoir of anaerobic glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 (open symbols) and H1022 (filled symbols) at a dilution rate of 0·10 h^{-1} and pH 5·0. Data are based on the values in the accompanying paper (Verduyn *et al.*, 1990).

Table 4. *Effect of culture pH on the energetics of anaerobic glucose-limited chemostat cultures of S. cerevisiae CBS 8066*

Growth was at $D = 0·10$ h^{-1}.

	Culture pH:			
	2·8	3·4	5·0	7·1
Glucose (g l^{-1})	1·85	0·10	0·07	0·12
Biomass (g l^{-1})	1·19	1·83	2·36	1·91
Cell yield (g g^{-1})	0·087	0·080	0·103	0·083
Ethanol (mM)	199	207	195	188
Glycerol (mM)	18·9	20·7	21·0	34·8
Acetate (mM)	1·4	0·6	0·5	9·9
Carbon recovery (%)	98	100	96	99
Y_{ATP} [g (mol ATP)$^{-1}$]	6·6	9·8	14·0	11·7

A maintenance coefficient (m_e; Pirt, 1965) could not be estimated from the results reported by Schatzmann (1975) for strain H1022, nor from our own data with this strain and *S. cerevisiae* CBS 8066. This is not surprising, since the protein content of the yeasts was strongly dependent on the dilution rate (Fig. 1). At low dilution rates, m_e is probably masked by the decrease in energy requirements for biomass formation; m_e is probably low anyway. Studies on the maintenance of different respiration-deficient mutants of *S. cerevisiae* (Watson, 1970; Rogers & Stewart, 1974) and for 'micro-aerobical' growth of *S. cerevisiae* (Rogers & Stewart, 1974) indicate an m_e of approximately 0·5 mmol ATP (g biomass)$^{-1}$ h^{-1}. The effect of such low values on Y_{ATP} is small and results in a Y_{ATP}^{max} of approximately 17 g biomass (mol ATP formed)$^{-1}$, if Y_{ATP} is taken as 16.

Since m_e could not be determined accurately, but is presumably small, it was decided to base all calculations

Table 3. *Effect of propionate on anaerobic glucose-limited chemostat cultures of S. cerevisiae CBS 8066*

Growth was at pH 5·0 and $D = 0·10$ h^{-1}.

	Propionate concentration in the reservoir (mM):*		
	7	17	25
Propionate (mM)	5·5	14·5	22·0
Glucose (g l^{-1})	0·16	0·38	0·52
Biomass (g l^{-1})	1·82	1·19	0·99
Cell yield (g g^{-1})	0·080	0·053	0·043
Ethanol (mM)	207	215	211
Glycerol (mM)	16·7	11·8	10·9
Carbon recovery (%)	97	98	95
Y_{ATP} [g (mol ATP)$^{-1}$]	9·6	5·9	4·9
Maintenance energy [mmol ATP$_{eq.}$ (g biomass)$^{-1}$ h^{-1}]	4·2	10·7	14·2

* At 30 mM-propionate the culture washed-out.

on an experimentally determined Y_{ATP} of 16. With a fixed value for Y_{ATP}, it is possible to quantify the effects of propionate in terms of ATP. In these calculations, it is assumed that Y_{ATP}^{max} is constant and identical to Y_{ATP} [16 g biomass (mol ATP formed)$^{-1}$], since no change in the biomass composition was observed. The calculated m_e (equation 2, Methods) required to compensate for uncoupling by propionate, acetate or succinate, is shown in Fig. 2. A linear correlation is apparent for the concentration of acid in the culture and m_e, with a calculated maximal value of 17 ± 2 mmol ATP$_{eq}$. (g biomass)$^{-1}$ h^{-1}. For *S. cerevisiae* H1022, the uncoupling effect of propionate (residual concentration 15 ± 1 mM) was also tested at dilution rates of 0·15 and 0·20 h^{-1}. This gave an m_e value of $10 \pm 1·5$ mmol ATP$_{eq}$. (g biomass)$^{-1}$ h^{-1}, similar to the value at $D = 0·10$ h^{-1} (Fig. 2). Thus the effect of propionate appears to be independent of the growth rate and can be regarded as a maintenance effect.

From the data in Fig. 2, the effect of uncoupling by the acetate produced in anaerobic cultures of *S. cerevisiae* CBS 8066 can be calculated. At $D = 0·10$ h^{-1}, 0·5 mM-acetate was found in the supernatant (Table 1). At this concentration the m_e due to uncoupling would amount to 0·3 mmol ATP$_{eq}$. (g biomass)$^{-1}$ h^{-1} (Fig. 2). The Y_{ATP} of *S. cerevisiae* CBS 8066, corrected for the effect of acetate, is then 14·6 (equation 2, Methods). This value is slightly different from that [$Y_{ATP} = 15·8 \pm 0·6$ g biomass (mol ATP)$^{-1}$] found for *S. cerevisiae* H1022, which shows negligible acetate formation but has a slightly lower protein content (Table 1). Thus, at pH 5·0, the difference in acetate production could at least partly explain the difference in Y_{ATP} between the two yeasts.

Effect of extracellular pH on the energetics of anaerobic growth

The effects of extracellular pH on the energetics of *S. cerevisiae* CBS 8066 and H1022 grown anaerobically at $D = 0·10$ h^{-1} are shown in Table 4 (pH values were adjusted without addition of extra acid). Below pH 2·6 $\pm$ 0·1, wash-out of both strains occurred. However, below pH 2·8 the pH could not be controlled with sufficient accuracy (within $\pm$ 0·05 pH units). This resulted in transient accumulation of glucose in the culture and in variable concentrations of biomass, ethanol etc. Therefore, true steady-states could not be established below this pH. Y_{ATP} showed an optimum at pH 5·0 to 5·5 (Fig. 3). Below this pH it decreased drastically, to reach a value of 6·6 at pH 2·8. A study of growth yields at different dilution rates indicated that the observed Y_{ATP} followed the equation $Y_{ATP} = m_e/\mu + 1/Y_{ATP}^{max}$ ($Y_{ATP}^{max} = 16$ at $D = 0·10$ h^{-1}). Thus, the effect of culture pH can be regarded as an effect on maintenance. m_e increased to a value as high as 9 mmol ATP$_{eq}$. (g biomass)$^{-1}$ h^{-1} (Fig.
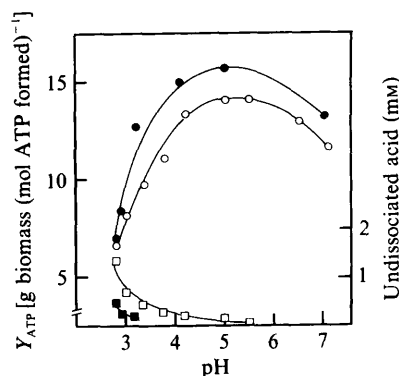

Fig. 3. Y_{ATP} (O, ●) and concentration of undissociated acetic acid (□, ■) as a function of culture pH during anaerobic glucose-limited growth of *S. cerevisiae* CBS 8066 (open symbols) and H1022 (filled symbols) at a dilution rate of 0·10 h^{-1}.
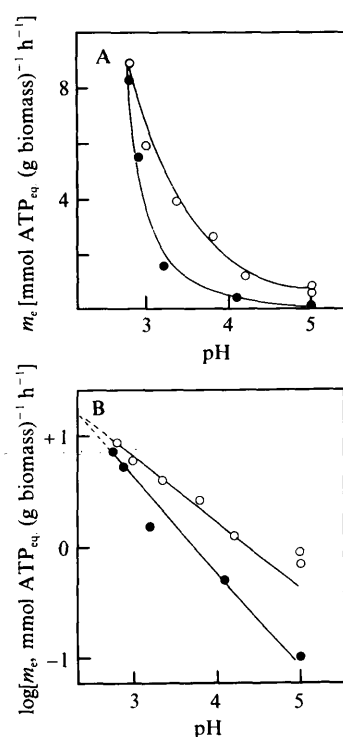

Fig. 4. A, Calculated m_e as a function of culture pH for anaerobic glucose-limited growth of *S. cerevisiae* CBS 8066 (O) and H1022 (●) at a dilution rate of 0·10 h^{-1}. Y_{ATP}^{max} (Y_{ATP} without uncoupling) was assumed to be 16. B, as A but with m_e plotted as a logarithm.

4A). It can be expected that uncoupling by acetic acid will increase with decreasing pH, due to an increased concentration of the undissociated acid. However, upon a decrease in culture pH from, for instance, 5 to 3·5, m_e for *S. cerevisiae* CBS 8066 increased fourfold (Fig. 4A), whereas the concentration of undissociated acetic acid (calculated from the Henderson–Hasselbach equation using a pK of 4·74) increased only twofold, from 0·14 to 0·3 mM (Fig. 3). Furthermore, Cássio *et al.* (1987), in a

Table 5. *Effect of propionate on the maintenance requirement of an anaerobic glucose-limited chemostat culture of S. cerevisiae H1022*

Growth was at $D = 0.10$ h^{-1}. The concentration of propionate in the medium reservoir was 5.0 mM.

pH	Propionate (mM)	Undissociated propionic acid (mM)	Y_{ATP} No acid	Y_{ATP} With acid	Maintenance requirement due to addition of acid* m_e	Maintenance requirement due to addition of acid* $m_e \cdot$ (mM undissociated acid)$^{-1}$
3.5	4.1	3.8	12.4	9.6	2.4	0.6
5.0	4.0	1.5	15.8	10.9	2.8	1.9

* m_e in mmol ATP$_{eq.}$ (g biomass)$^{-1}$ h^{-1}.

study based on uptake of labelled weak acids, showed that in *S. cerevisiae* the permeability of the plasma membrane for undissociated weak acids decreases with decreasing pH over a pH range of 5.5 to approximately 3. This was confirmed in our study: addition of (non-metabolizable) propionate to the medium reservoir of *S. cerevisiae* H1022 at pH 3.5 resulted in a considerably lower m_e (expressed in terms of the concentration of undissociated acid) than at pH 5.0 (Table 5). Thus, uncoupling by a given amount of undissociated acetic acid at a given pH cannot be directly related to the effect at a lower pH, since uncoupling will decrease significantly with decreasing culture pH. It thus seems likely that (at least in the region below pH 5) the culture pH has an effect on Y_{ATP} that is, to a large extent, independent of the presence of acetic acid. The increased m_e at low pH must then be mainly ascribed to the increased proton gradient across the plasma membrane resulting in increased passive proton uptake rate. Plotting log m_e against culture pH results in approximately straight lines (Fig. 4B). This allows extrapolation to pH 2.6 ± 0.1, the region in which wash-out always occurred. The results suggest that the maximal m_e will be 12 ± 2 mmol ATP$_{eq.}$ (g biomass)$^{-1}$ h^{-1}.

Discussion

Literature data on the anaerobic energetics of yeasts

Y_{ATP}, calculated for yeasts from batch experiments, averages 10.5 to 11 g biomass (mol ATP formed)$^{-1}$ (Bauchop & Elsden, 1960; Kormančiková *et al.*, 1969). This is probably an underestimate of about 10%, as no allowance has been made for the energetic consequences of glycerol production. The usual method to determine Y_{ATP} in batch cultures involves measurement of biomass and metabolite concentrations at the end of the growth curve, where glucose is completely consumed. However,

this method is subject to a number of uncertainties, notably the fact that a limitation in the medium (other than the energy source) will not be readily apparent. Such a nutrient limitation seems the most likely explanation for the decline in Y_{ATP} observed by Haukeli & Lie (1971) during the course of anaerobic batch experiments with various yeasts. A nutrient limitation may also explain the very low Y_{ATP} observed in anaerobic glucose-limited chemostat cultures of *S. cerevisiae* CBS 426 by Dekkers *et al.* (1981). The aerobic Y_{ATP} for this organism was twice as high as the anaerobic value. The authors suggest that this difference may have been caused by differences in maintenance energy. This, however, is unlikely: the maintenance requirements of yeasts grown under energy limitation are generally low (Atkinson & Mavituna, 1983) and do not affect Y_{ATP} significantly. Rather, the explanation for the large difference must be sought in the fact that Dekkers *et al.* (1981) did not add an unsaturated fatty acid to the growth medium during anaerobic cultivation. As shown in the preceding paper (Verduyn *et al.*, 1990), suboptimal concentrations of Tween 80 result in a drastic decrease in Y_{ATP}.

The high value [approximately 16 g biomass (mol ATP)$^{-1}$; (Table 1)] obtained in this study for the evaluation of the energetics of aerobic growth of yeasts. In many reviews a Y_{ATP} of 10.5 during anaerobic growth has been used as the basis for calculation of the P/O-ratio for aerobic growth. This results in values for the P/O-ratio of approximately 1.8 (e.g. Barford & Hall, 1981; Andrews, 1989). However, if a value of 16 is used, with a cell yield of 0.5 g cells (g glucose)$^{-1}$ (Postma *et al.*, 1989), the 'effective' P/O-ratio becomes approximately 1.0.

Energetic consequences of proton influx

It is generally assumed that uncoupling by weak organic acids is a consequence of the influx of protons from the medium to the cytosol by diffusion of the undissociated

acid (Krebs *et al.*, 1983; Warth, 1989; Viegas *et al.*, 1989). To maintain the internal pH near neutrality, and prevent dissipation of the protonmotive force, protons must be pumped out via the plasma membrane ATPase, requiring the hydrolysis of ATP. When the influx of protons exceeds the capacity of the ATPase, acidification of the cytosol will occur, followed by cell death. Basically similar suggestions have been made by Warth (1988) and Viegas *et al.* (1989). Furthermore, Warth (1977, 1988) has surmised that extra energy might be expended for the expulsion of the anionic forms of weak acids from the cytosol to the medium, at least in the case of benzoate. In this respect one can envisage a system in which the anion leaves the cell in symport with a cation such as potassium.

To explain our results, a major role must be attributed to the plasma membrane ATPase. This enzyme is indispensible for yeast growth (Serrano *et al.*, 1986). It acts as a proton pump (for a review see Goffeau & Slayman, 1981) to create a proton gradient across the plasma membrane, which is used to drive uptake of nutrients (Vallejo & Serrano, 1989). The enzyme has also been implicated in regulation of the internal pH (Eraso & Gancedo, 1987; Eraso *et al.*, 1987), especially during growth in acidic environments. The results in Table 3 suggest that, once m_e increases to more than 17 mmol $ATP_{eq.}$ (g biomass)$^{-1}$ h^{-1}, anaerobic growth is not possible. This could be due either to saturation of the plasma membrane ATPase (i.e. a limiting amount of enzyme protein) or to a limiting supply of ATP. The latter possibility is not unlikely. It has been shown that the maximal pyruvate decarboxylase activity – which probably determines the maximal ethanol production rate (Postma *et al.*, 1989) – in *S. cerevisiae* CBS 8066 is approximately 1·2 μmol min^{-1} (mg protein)$^{-1}$, under aerobic (Postma *et al.*, 1989) as well as under anaerobic conditions (Verduyn *et al.*, 1990). With a dry weight/soluble protein ratio of 3:1, this pyruvate decarboxylase activity is sufficient for an ethanol flux of 1·2 × 0·33 × 60 = 24 mmol (g biomass)$^{-1}$ h^{-1}, equivalent to an ATP-production rate of 24 mmol (g biomass)$^{-1}$ h^{-1}. Synthesis of cell material requires an ATP flux ($q_{ATP} = \mu/Y_{ATP} = 0·10/16$) of 6·25 mmol ATP (g biomass)$^{-1}$ h^{-1}. With an m_e of 17, this results in a total ATP flux of 23 mmol (g biomass)$^{-1}$ h^{-1}, which almost equals the maximal ATP production rate. This calculation indicates that the fermentation rate limits the supply of ATP for maintenance purposes – above that required for anabolic reactions – to a value of approximately 17 mmol $ATP_{eq.}$ (g biomass)$^{-1}$ h^{-1}. This value is approached when sufficient weak acid is present, or when the culture pH is below 2·8. If the maintenance requirement increases further, sufficient ATP cannot be provided and acidification of the cytosol will occur, followed by cell death.

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