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

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The physiology of *Saccharomyces cerevisiae* CBS 8066 was studied in anaerobic glucose-limited chemostat cultures in a mineral medium supplemented with ergosterol and Tween 80. The organism had a μ_{max} of 0·31 h^{-1} and a K_s for glucose of 0·55 mM. At a dilution rate of 0·10 h^{-1}, a maximal yield of 0·10 g biomass (g glucose)$^{-1}$ was observed. The yield steadily declined with increasing dilution rates, so a maintenance coefficient for anaerobic growth could not be estimated At a dilution rate of 0·10 h^{-1}, the yield of the *S. cerevisiae* strain H1022 was considerably higher than for CBS 8066, despite a similar cell composition. The major difference between the two yeast strains was that *S. cerevisiae* H1022 did not produce acetate, suggesting that the observed difference in cell yield may be ascribed to an uncoupling effect of acetic acid. The absence of acetate formation in H1022 correlated with a relatively high level of acetyl-CoA synthetase. The uncoupling effect of weak acids on anaerobic growth was confirmed in experiments in which a weak acid (acetate or propionate) was added to the medium feed. This resulted in a reduction in yield and an increase in specific ethanol production. Both yeasts required approximately 35 mg oleic acid (g biomass)$^{-1}$ for optimal growth. Lower or higher concentrations of this fatty acid, supplied as Tween 80, resulted in uncoupling of dissimilatory and assimilatory processes.

Introduction

Saccharomyces cerevisiae is one of the few yeasts capable of anaerobic growth, although only in the presence of added sterol and unsaturated fatty acids. In yeasts, these compounds cannot be synthesized in the absence of oxygen (Andreasen & Stier, 1953, 1954).

The physiology of *S. cerevisiae* in *aerobic* glucose-limited chemostat cultures has been the subject of many studies. *S. cerevisiae* displays the so-called Crabtree effect: alcoholic fermentation in the presence of oxygen when the glucose concentration exceeds a certain threshold value (Barford *et al.*, 1981; Fiechter *et al.*, 1981; Verduyn *et al.*, 1984*b*). In glucose-limited chemostat cultures this effect is apparent at high dilution rates (Postma *et al.*, 1989*a*), and also after a glucose pulse to an aerobic culture pregrown at a low dilution rate (van Urk *et al.*, 1988). In the presence of excess sugar, aerobic fermentation of glucose to ethanol provides a substantial part of the energy required for the formation of biomass. At first sight there is a considerable similarity between aerobic chemostat growth at high dilution rates and anaerobic growth: both conditions are characterized by high glucose and ethanol fluxes, low yields as compared to fully respiratory growth, and appearance of various byproducts. The latter may affect metabolism, as shown for weak acids during aerobic glucose-limited chemostat growth of *S. cerevisiae* CBS 8066 (Postma *et al.*, 1989*a*). It is, however, difficult to compare aerobic and anaerobic growth in quantitative terms, since little information is available on the anaerobic physiology of yeasts growing under defined conditions, most information having been obtained from batch cultures.

In this paper, some fundamental parameters for anaerobic growth in chemostat cultures of two *S. cerevisiae* strains are presented. Particular attention is paid to the effect of weak acids on metabolic activity.

Methods

Micro-organisms and growth conditions. Saccharomyces cerevisiae CBS 8066 was obtained from the Centraal Bureau voor Schimmelcultures, Delft, the Netherlands. *S. cerevisiae* H1022 was kindly donated by Dr O. Käppeli, ETH Zürich, Switzerland. This strain has now been included in the CBS collection as CBS 7336. Both yeasts were maintained on agar slants at 4 °C

Anaerobic chemostat cultivation of the yeasts was done at 30 °C in an Applikon fermenter with a working volume of 1 litre and a stirring speed of 600 r.p.m. The pH was automatically controlled at the desired value by titration with 2 M-KOH. Cultivation was at pH 5·0, unless mentioned otherwise. The condenser was connected to a cryostat and cooled at 2 °C. The tubing on the entire fermenter set-up (including medium- and waste-reservoirs) consisted of Norprene (Cole-Parmer Corp.). The fermenter and the medium reservoir (with a magnetic stirrer) were continuously sparged with certificated ultra-pure nitrogen (Air Products), containing less than 5 p.p.m. oxygen, at a flow rate of 0·5 l min^{-1}.

The mineral medium, supplemented with vitamins and trace elements, was prepared according to Bruinenberg *et al.* (1983*a*), except that the concentration of $NaMoO_4.2H_2O$ was increased tenfold. Vitamins were filter-sterilized and added after heat sterilization of the medium. Ergosterol and Tween 80 were dissolved in pure ethanol and steamed at 100 °C for 10 min before being added to the medium to final concentrations of 10 mg l^{-1} and 420 mg l^{-1}, respectively. The concentration of ethanol in the medium reservoir was 1 mM. The glucose concentration in the reservoir (S_r) was 23 g l^{-1}. Ammonium sulphate (5 g l^{-1}) was used as nitrogen source.

Calculation of carbon recovery. Carbon recoveries were calculated from the production of biomass, metabolites and CO_2. CO_2 was not measured, but calculated from the concentrations of ethanol and acetate and the amount of biomass. CO_2 production during biomass formation was calculated according to Bruinenberg *et al.* (1983*b*) on the basis of the cell composition under the various growth conditions. The carbon recovery in all experiments was 95 to 102%, with a mean of 97%.

Determination of dry weight. Dry weights of cultures were determined using a magnetron oven and 0·45 μm filters as described previously (Postma *et al.*, 1989*a*). This procedure yielded the same results as drying at 80 °C on filters or in weighing-flasks. Parallel samples varied by less than 2%.

Analysis of metabolites. Acetate, acetaldehyde, glycerol, succinate and glucose were determined with Boehringer test-kits 148261, 668613, 148270, 176281 and 676543, respectively. Ethanol was determined colorimetrically by the following procedure. A 50 μl sample containing 0·05 to 0·5 g ethanol l^{-1} was added to 2 ml of a solution (in 0·1 M-potassium phosphate buffer, pH 7·5) containing: 4-aminoantipyrine, 0·4 mM; 4-hydroxybenzenesulphonic acid, 25 mM; horseradish peroxidase, 2 Units: and alcohol oxidase (prepared according to Verduyn *et al.*, 1984*a*), 5 Units. The mixture was incubated at room temperature for 45 min and the absorbance at 500 nm was then read with a spectrophotometer. Pyruvate was determined according to Postma *et al.* (1989*a*), as well as by HPLC as described below. Both procedures gave the same results. Organic acids were determined by HPLC on a HPX-87H column (300 × 7·8 mm; Bio-Rad) at room temperature. The column was eluted with 5 mM-H_2SO_4 at a flow rate of 0·6 ml min^{-1}. Detection was by means of a Waters 441 UV-meter at 210 nm, coupled to a Waters 741 data module. Peak areas were linearly proportional to concentrations. The detection limits (with a 20 μl injection volume) for acetate, pyruvate, succinate and propionate were below 50 μM.

Protein determinations. Protein in cell-free extracts was determined by the Lowry method. The protein content of whole cells was assayed by a modified biuret method. A fresh 10 ml sample of the culture (2 to 3 g dry wt l^{-1}) was centrifuged, and the yeast was washed twice with distilled water and resuspended in 5 ml of water. The concentrate was boiled in 1 M-KOH (final concentration) for 10 min and subsequently cooled on ice. $CuSO_4.5H_2O$ was then added to a final concentration of 25 mM. After 5 min, the mixture was centrifuged in an Eppendorf bench-top centrifuge (13000 r.p.m.) for 2 min and the absorbance of the supernatant was read at 550 nm. In both assays, BSA (fatty-acid free; Sigma) was used as a standard.

Carbon, hydrogen and nitrogen analysis. Fresh cell samples were washed twice with distilled water and dried at 70 °C for 72 h. The C-, H- and N-contents were then determined with an Elemental Analyser 240B (Perkin-Elmer). Deviations in the C-, H- and N-contents for separate samples were less than 0·5, 0·2 and 0·2% (absolute values), respectively. From the literature (Oura, 1972; Aiking & Tempest, 1976; Barford & Hall, 1979*b*; Maiorella *et al.*, 1984) it can be derived or has been shown that carbon, hydrogen, nitrogen and oxygen together make up approximately 94% of the dry weight of biomass. Hence, from the C-, H- and N-contents (determined experimentally), the oxygen content can be calculated.

Enzyme assays. Enzyme assays were done with fresh cell-free extracts at 30 °C with a Hitachi model 100-60 spectrophotometer. Reaction rates were proportional to the amount of enzyme added. Preparation of cell-free extracts and assays of pyruvate decarboxylase (EC 4.1.1.1, alcohol dehydrogenase (EC 1.1.1.1), acetaldehyde dehydrogenase (NAD^+- and $NADP^+$-dependent; EC 1.2.1.5 and EC 1.2.1.4, respectively), acetyl-CoA synthetase (EC 6.2.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were done according to Postma *et al.* (1989*a*). Cytochrome *c* peroxidase (EC 1.11.1.5) and catalase (EC 1.11.1.6) were assayed according to Verduyn *et al.* (1988). Extracts from aerobically grown cells ($D = 0{\cdot}10\ h^{-1}$) were used as a control for the latter two enzymes. One Unit is defined as the amount of enzyme catalysing the conversion of 1 μmol substrate min^{-1}. Specific activities are expressed as Units (mg protein)$^{-1}$.

Chemicals. Enzymes used in metabolite assays were obtained from Boehringer. 4-Hydroxybenzenesulphonic acid and 4-aminoantipyrine were obtained from BDH and Aldrich, respectively. Tween 80 was obtained from E. Merck.

Results

Requirements for unsaturated fatty acids

In contrast to earlier reports (Andreasen & Stier, 1953, 1954), strictly anaerobic growth has been reported for *S. cerevisiae* in batch cultures in the absence of added sterols and fatty acids (Macy & Miller, 1983). In our experiments, growth without added fatty acid was possible at $D = 0{\cdot}10\ h^{-1}$ (pH 5·0) for both *S. cerevisiae* CBS 8066 and H1022. Omission of Tween 80 (a source of oleic acid) from the growth medium, however, led to a drastic reduction in biomass (Fig. 1) and a corresponding increase in ethanol production (not shown). Furthermore, the residual glucose concentration in the culture was sixfold higher in the absence of Tween 80 as compared to standard cultivation conditions with 420 mg Tween 80 l^{-1} (Fig. 1). Addition of increasing amounts of Tween 80 to *S. cerevisiae* H1022 (at a fixed concentration of ergosterol) resulted in an increase in the biomass from 1·80 g l^{-1} without Tween 80, to a maximum of 2·64 g l^{-1} at a Tween 80 concentration of 420 mg l^{-1}. This corresponds to an oleic acid content of approximately 3·5% of dry weight, when it is assumed that all oleate provided is incorporated into biomass. When the concentration of Tween 80 was increased further, there was a progressive decrease in biomass; at a Tween 80 concentration of 1 g l^{-1}, the biomass concen-

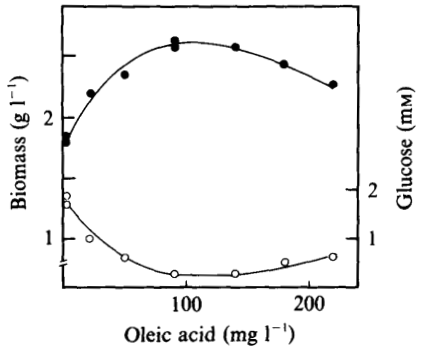

Fig. 1. Effect of oleate (added as Tween 80) on the formation of biomass (●) and the residual glucose concentration (○) during glucose-limited chemostat growth of *S. cerevisiae* H1022 at a dilution rate of 0·10 h^{-1} (pH 5·0).

tration declined to 2·30 g l^{-1} (Fig. 1). For *S. cerevisiae* CBS 8066, a similar pattern was observed, but in this case the amount of biomass was slightly (2%) higher at 840 mg Tween 80 l^{-1} then at 420 mg l^{-1}, probably indicating that CBS 8066 has a somewhat higher fatty acid content than H1022. From literature data (Oura, 1972; Jones & Greenfield, 1987) it can be concluded that the lipid fraction of plasma membranes makes up 2·5 to 5% of the total dry weight of biomass, whereas the total lipid content is somewhat higher. Therefore, the values obtained here are not unrealistic. Tripling the concentration of ergosterol had no effect on the yield. This is not surprising, since the sterol content of yeast is approximately 0·2% of dry weight (Munoz & Ingledew, 1989).

It should be stressed that strict anaerobic conditions, especially in a complicated system such as a chemostat, are not possible, unless oxygen-trapping chemicals are added to the growth media. Since these agents (for example sulphite) are known to affect the physiology of yeasts they were not added. Thus it is likely that minute amounts of oxygen could still reach the fermenter and might be used in the synthesis of unsaturated fatty acids. If it is assumed that all the fatty acid (3·5% of dry weight) consists of oleate (a mono-unsaturated fatty acid) the oxygen requirement should be 1·0 mmol O_2 (mmol oleate)$^{-1}$ [or 124 μmol O_2 (g biomass)$^{-1}$]. Thus, at a dilution rate of 0·10 h^{-1}, diffusion of oxygen into the culture at a rate of only 31 μmol h^{-1} would be sufficient to meet the requirement for synthesis of unsaturated fatty acid. Therefore, the growth observed in the absence of added fatty acid (Fig. 1) does not prove that oxygen is not required in the biosynthesis of unsaturated fatty acids.

Biomass and metabolite formation as a function of the dilution rate

S. cerevisiae CBS 8066 was grown anaerobically under glucose limitation at dilution rates (D) from 0·05 to

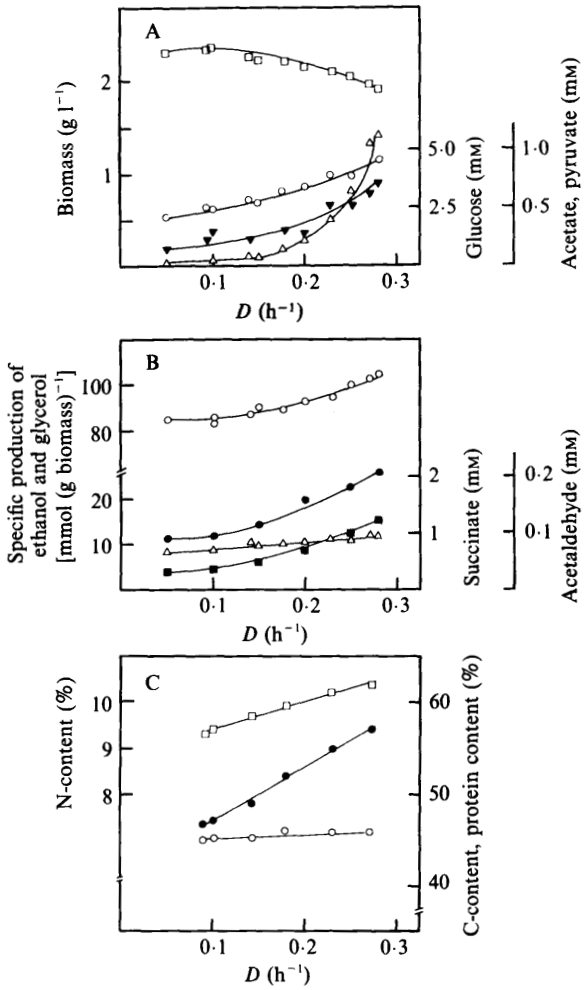

Fig. 2. A, biomass (□) and concentrations of glucose (△), acetate (○) and pyruvate (▼) in anaerobic glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 as a function of the dilution rate (pH 5·0). B, specific production of ethanol (○) and glycerol (△) and concentrations of acetaldehyde (■) and succinic acid (●); conditions as in A. C, carbon (○), nitrogen (□) and protein (●) content of biomass; conditions as in A,

0·28 h^{-1}, the highest dilution rate at which a steady state could be attained. The residual glucose concentration in the fermenter followed classical Monod kintics. From a Hanes' plot (not shown), a μ_{max} of 0·31 h^{-1} and a K_s for glucose of 0·55 mM were calculated. The amount of biomass decreased steadily with increasing dilution rate (Fig. 2A). The amount of ethanol produced per unit of biomass increased in parallel from 87 ± 5 to 105 ± 5 and that of glycerol from 9·1 ± 0·5 to 11·6 ± 0·5 mmol (g biomass)$^{-1}$, respectively (Fig. 2B). Acetate was also produced at all dilution rates; its concentration increased from 0·4 ± 0·1 mM at $D = 0{\cdot}05$ h^{-1} to 0·9 ± 0·2 mM at $D = 0{\cdot}28$ h^{-1} (Fig. 2A). Other minor products, as detected by HPLC, were: pyruvate (increasing from 0·13 ± 0·05 to 0·7 ± 0·1 mM; fig. 2A); succinate (increas-

Table 1. *Biomass concentration, protein content of biomass, and metabolite concentrations during anaerobic glucose-limited chemostat growth of S. cerevisiae CBS 8066 and H1022*

Growth was at $D = 0{\cdot}10\ h^{-1}$ and pH 5·0. The glucose concentration in the reservoir was $23\ g\ l^{-1}$.

			Metabolite concn (mM)					
Strain	Biomass ($g\ l^{-1}$)	Protein (%)	Residual glucose	Ethanol	Glycerol	Acetate	Succinate	Pyruvate
CBS 8066	2·36	47	0·20	195	21·2	0·50	0·9	0·20
H1022	2·64	45	0·28	191	23·6	0·04	1·1	0·15

ing from $1 \pm 0{\cdot}2$ to $2 \pm 0{\cdot}3$ mM; Fig. 2B); and malate (decreasing from $0{\cdot}8 \pm 0{\cdot}1$ to $0{\cdot}2 \pm 0{\cdot}1$ mM). Moreover, low concentrations (<0·15 mM) of fumarate and 2-oxoglutarate, as well as acetaldehyde (40 ± 10 to 120 ± 20 μM; Fig. 2B), were also found.

The highest biomass output, at $D = 0{\cdot}10\ h^{-1}$, was $2{\cdot}36 \pm 0{\cdot}04$ g biomass l^{-1}, corresponding to a yield (Y) of 0·103 g biomass (g glucose)$^{-1}$. The protein content at this dilution rate was $47 \pm 2\%$ (w/w), but it increased to $57 \pm 2\%$ at $D = 0{\cdot}28\ h^{-1}$ (Fig. 2C).

When *S. cerevisiae* H1022 was grown at $D = 0{\cdot}10\ h^{-1}$, its biomass output was significantly higher than for CBS 8066 at the same dilution rate. Furthermore, acetate production was negligible during anaerobic growth of H1022 (Table 1). Our data confirm the original observations of Schatzmann (1975) with this strain. The protein content of H1022, however, was similar to that of CBS 8066. Also the patterns and quantities of minor byproducts (pyruvate, succinate etc.) were similar for both strains (Table 1).

Biomass and metabolite formation as a function of pH

When *S. cerevisiae* CBS 8066 was cultivated at a constant dilution rate ($D = 0{\cdot}10\ h^{-1}$) over a wide pH range, an optimum in biomass production was observed at pH 5 to 5·5 (Fig. 3). Below pH 2·7, wash-out occurred. The residual glucose and acetate concentrations were low at the pH where biomass output was maximal. At pH values above 5·5, a pronounced increase in the concentrations of acetate and glycerol was observed (Fig. 3). It can be calculated that the production of glycerol per amount of biomass was relatively constant between pH 3·0 and 5·5. The protein content was constant at $47 \pm 2\%$ over the entire pH range. *S. cerevisiae* H1022 behaved similarly and also washed-out below pH 2·7.

Effect of added weak acids

Since weak acids act as uncouplers in yeasts (Postma *et al.*, 1989*a*) and because of the significant difference

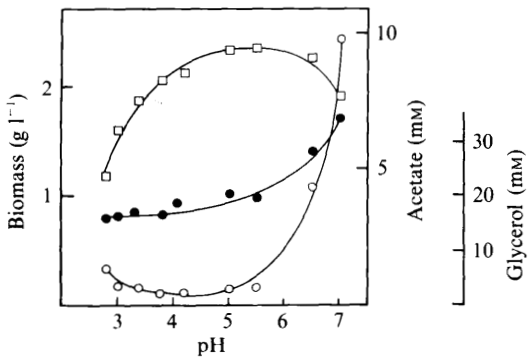

Fig. 3. Effect of culture pH on formation of biomass (□), and of acetate (○) and glycerol (●), in anaerobic glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 at a dilution rate of $0{\cdot}10\ h^{-1}$.

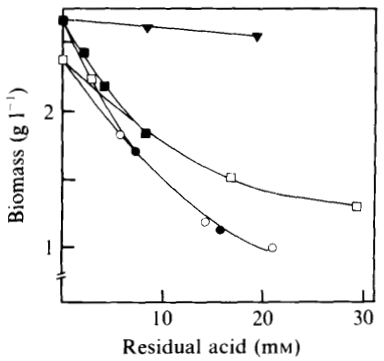

Fig. 4. Effect of addition of acids to the medium feed on biomass formation in glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 (open symbols) and H1022 (filled symbols) at a dilution rate of $0{\cdot}10\ h^{-1}$ (pH 5·0). Data on the *x*-axis are residual culture concentrations of acetate (□, ■), propionate (○, ●) and succinate (▼).

between the two yeasts with respect to acetate production (Table 1), it was of interest to study the effect of weak acids on their physiology. Addition of acetate to the medium reservoir of *S. cerevisiae* CBS 8066 resulted in a progressive drop in the biomass concentration with increasing additions of acetate.

Propionate had a similar, but more pronounced effect. When propionate or acetate was added to H1022, the results were virtually identical to those obtained with

Table 2. *Comparison of theoretical accumulation and experimental accumulation of acetate and propionate by S. cerevisiae CBS 8066*

The theoretical accumulation factor was calculated assuming a cytoplasmic pH of 7·0. From the experimentally determined accumulation factor, the actual cytosolic pH was then calculated.

Reservoir concn (mM)		Residual concn in the culture (mM)	Theoretical accumulation factor	Experimental accumulation factor		Cytosolic pH (mean)
Acetate	3·5	2·5		91		
	20·0	16·0	65	82	84	7·1
	35·5	29·5		78		
Propionate	7·0	5·5		71		
	17·0	14·5	58	72	71	7·1
	25·0	22·0		69		

CBS 8066 (Fig. 4). Ethanol production per unit of biomass increased with increasing concentrations of acid. However, the specific glycerol production was not significantly affected. In all cases, the residual glucose concentration increased with increasing addition of acetate or propionate (data not shown).

If it is assumed that weak acids are distributed according to the ΔpH (i.e. the difference between the intra- and the extracellular pH), the accumulation factor of the acid in the cell can be calculated. The expression log(accumulation factor) = $pH_{in} - pH_{out}$ is often used (e.g. Cássio *et al.*, 1987), but is only valid if the fraction of undissociated acid is small as compared to the total amount of acid, i.e. when the external pH is at least 1 unit higher than the pK. When a considerable fraction of the acid is in the undissociated form, the following expression is applicable

$$pH_{in} = \log[(\text{total acid}_{in}/\text{total acid}_{out})\,(10^{pK} + 10^{pH_{out}} - 10^{pK}]$$

In a more accessible form this can be written as

$$\frac{\text{total acid}_{in}}{\text{total acid}_{out}} = \frac{10^{(pH_{in} - pK)} + 1}{10^{(pH_{out} - pK)} + 1}$$

With an estimated intracellular pH of 7 and an extracellular pH of 5, the theoretical accumulation factor for acetate (pK = 4·74) would be 65. The average experimental accumulation factor of acetate added to *S. cerevisiae* CBS 8066, as calculated from the difference between the reservoir concentration and the residual concentration in the fermenter in steady state was 84 ± 10 (Table 2). In this calculation, it was assumed that the cytosolic volume was 2 ml (g cells)$^{-1}$ (Valle *et al.*, 1986; Warth, 1988). The accumulation factor was independent of the amount of acetate added (Table 2). The corresponding intracellular pH according to the above equation would be 7·1 ± 0·1. In a similar way, the accumulation factor of propionate (pK = 4·87) was calculated as 71 ± 10 (indicating an intracellular pH of 7·1). For *S. cerevisiae* H1022 slightly lower accumulation factors were calculated – 65 and 59 for acetate and propionate, respectively – both corresponding to an intracellular pH of 7·0. Literature data on the cytoplasmic pH of yeasts indicate values of 6·8–7·2 (Navon *et al.*, 1979; Nicolay *et al.*, 1982; Warth, 1988; van Urk *et al.*, 1989).

Enzyme activities and product formation

In *S. cerevisiae* CBS 8066 and H1022, the activities of enzymes of the fermentative pathway (pyruvate decarboxylase and alcohol dehydrogenase) were similar. The activity of glucose-6-phosphate dehydrogenase, a key enzyme in the hexose monophosphate pathway, was also the same in both yeasts (Table 3). However, important differences were observed for the acetate-producing and acetate-consuming enzymes: in H1022 the activity of the acetaldehyde dehydrogenases was three times lower than in CBS 8066, and the activity of acetyl-CoA synthetase four times higher (Table 3). This may explain why H1022 does not excrete detectable amounts of acetate (Table 1).

In addition, the typical 'aerobic' enzymes cytochrome *c* peroxidase and catalase, which are known to appear at very low oxygen concentrations (Chantrenne, 1955; Oura, 1972; Lee & Hassan, 1986) were assayed. No activity of these enzymes was detectable in anaerobic cultures.

Theoretical aspects of metabolite formation

When the cell yield and the assimilation equation for the formation of biomass are known, the amounts of ethanol, glycerol and CO_2 formed (neglecting other minor byproducts) can be predicted. The assimilation equation follows from summation of all the biosynthetic routes leading to the formation of biomass (Oura, 1972;

Table 3. *Enzyme activities in anaerobic glucose-limited chemostat cultures of S. cerevisiae CBS 8066 and H1022*

Growth was at $D = 0{\cdot}10\ h^{-1}$.

	Enzyme activity [Units (mg protein)$^{-1}$]					
Strain	Alcohol dehydrogenase	Pyruvate decarboxylase	Glucose-6-phosphate dehydrogenase	NAD^+-acetaldehyde dehydrogenase	$NADP^+$-acetaldehyde dehydrogenase	Acetyl-CoA synthetase
CBS8066	2·4	1·2	0·21	0·068	0·053	0·008
H1022	1·6	1·2	0·29	0·022	0·023	0·034

Table 4. *Production (+) or consumption (−) of reducing equivalents and CO_2 and glucose requirement for biomass formation during anaerobic chemostat growth of S. cerevisiae CBS 8066*

Growth was at $D = 0{\cdot}10\ h^{-1}$. The cell composition was taken as 47% protein, 37·5% carbohydrate, 7% RNA, 3·5% fatty acid and 5% ash (accompanying paper: Verduyn *et al.*, 1990).

Cell constituent	NADH [mmol (100 g biomass)$^{-1}$]	NADPH	CO_2	Glucose requirement (mmol for formation of 100 g of biomass)
Protein	+1033	−626	+273	420
Carbohydrate	0	0	0	231
RNA	+69	−22	−12	18
Fatty acid*	0	0	0	0
Total	+1102	−648	+261	+669

* Assumed to be taken up from the medium.

Bruinenberg *et al.*, 1983*b*). The net production or consumption of reducing equivalents and the glucose requirement for the synthesis of the main cell constituents are given in Table 4. It has been assumed that all fatty acid in the biomass is oleic acid taken up from the medium and that no *de novo* synthesis occurs. Bulder & Reinink (1974) showed that during anaerobic growth the majority of the fatty acids in *S. cerevisiae* are unsaturated and that their composition closely mirrors that of the fatty acid(s) supplied in the growth medium.

With the data presented in Table 4, the equation for the formation of 100 g of biomass (molecular mass, including ash, of 100) during anaerobic growth of *S. cerevisiae* CBS 8066 at $D = 0{\cdot}10\ h^{-1}$ can be constructed

$$669\ C_6H_{12}O_6 + 630\ NH_3 + 648\ NADPH + 648H^+ + 1102\ NAD^+ \rightarrow 1000\ C_{3{\cdot}75}H_{6{\cdot}6}N_{0{\cdot}63}O_{2{\cdot}10} + 261\ CO_2 + 648\ NADP^+ + 1102NADH + 1102\ H^+ + 1105\ H_2O \quad (1)$$

Furthermore, 54 mmol glucose (648/12) are required for the generation of NADPH with a concomitant production of 324 mmol CO_2 (for simplicity it is assumed that glucose is completely oxidized to CO_2 in the hexose monophosphate pathway, with a yield of 12 NADPH per glucose; cf. Bruinenberg *et al.* (1983*b*)

$$54\ C_6H_{12}O_6 + 648\ NADP^+ + 324\ H_2O \rightarrow 648\ NADPH + 648\ H^+ + 324\ CO_2 \quad (2)$$

Moreover, in order to close the redox balance for the co-enzyme system NAD^+/NADH, yeasts synthesize glycerol during anaerobic growth (Holzer *et al.*, 1963; Gancedo *et al.*, 1968; Oura, 1977) according to

$$551\ C_6H_{12}O_6 + 1102\ NADH + 1102\ H^+ \rightarrow 1102\ C_3H_8O_3 + 1102\ NAD^+ \quad (3)$$

From (1), (2) and (3), it follows that overall 723 + 551 = 1274 mmol glucose are required in anabolic reactions to form 100 g of biomass

$$1274\ C_6H_{12}O_6 + 630\ NH_3 \rightarrow 1000\ C_{3{\cdot}75}H_{6{\cdot}6}N_{0{\cdot}63}O_{2{\cdot}10} + 1102\ C_3H_8O_3 + 585\ CO_2 + 781\ H_2O \quad (4)$$

With a cell yield of 0·103 g (g glucose)$^{-1}$ (Table 1) for CBS 8066, 5394 mmol glucose are required in total, of which 5394 − 1274 = 4120 are consumed in dissimilation for the production of 100 g of biomass, with concomitant formation of 2 × 4120 = 8240 mmol ethanol

$$4120\ C_6H_{12}O_6 \rightarrow 8240\ C_2H_6O + 8240\ CO_2 \quad (5)$$

Thus, on a theoretical basis formation of biomass of the composition indicated in Table 4 would lead to the equation

$$5394\ \text{glucose} + 630\ NH_3 \rightarrow 100\ \text{g biomass} + 8240\ \text{ethanol} + 1102\ \text{glycerol} + 8825\ CO_2 + 781\ H_2O \quad (6)$$

This fits reasonably well with the observed values. From Table 1 it follows that for *S. cerevisiae* CBS 8066.

$$5394\ \text{glucose} + 630\ NH_3 \rightarrow 100\ \text{g biomass} + 8263\ \text{ethanol} + 898\ \text{glycerol} \quad (7)$$

From the data presented in Table 4, it follows that biosynthesis of protein (or, more correctly, amino acids) is by far the most important NADH-yielding process. It is thus expected that variations in protein content will be reflected in the amount of glycerol formed. Following an increase in dilution rate from 0·09 to 0·28 h^{-1}, the protein content increased from 47 to 57% (Fig. 2C). If the RNA level is assumed to increase from 7 to 10% (Furukawa *et al.*, 1983), it can be calculated from Table 4 that this shift in dilution rate should result in a 23% increase in surplus NADH. Over this range of dilution rates, the specific glycerol production increased from 9·1 ± 0·6 to 11·6 ± 0·6 mmol (g biomass)$^{-1}$, an increase of 27%. This confirms the role of glycerol as a redox sink under anaerobic conditions. Furthermore, during cultivation at high pH, leading to an increase in the acetate production, a considerable increase in glycerol production was observed (Fig. 3). This indicates that acetate production proceeds mainly via NAD^+-acetaldehyde dehydrogenase, resulting in net production of two NADH per acetate formed (van Dijken & Scheffers, 1986).

Discussion

Effect of fatty acids on the anaerobic physiology of S. cerevisiae

Yeasts are unable to synthesize unsaturated fatty acid or ergosterol in the absence of oxygen (Andreasen & Stier, 1953, 1954: Kates, 1966). The amount of unsaturated fatty acid declines when aerobically grown cells are transferred to anaerobic conditions in the absence of added sterol and fatty acid (Kováč *et al.*, 1967; Gordon & Stewart, 1972). Anaerobic growth in the absence of added sterols and fatty acid has been reported for *S. cerevisiae*, but this was accompanied by a reduction in both μ_{max} and growth yield, and by long lag phases (Macy & Miller, 1983). In our experiments also, growth was observed in the absence of added fatty acids; this was most probably due to the experimental set-up in which the entrance of minute amounts of oxygen cannot be avoided. The cell yield was, however, lower than when Tween 80 (oleic acid) was included in the growth medium. This lower cell yield may be explained in two ways. Fatty acid limitation may result in altered membrane properties (cf. Jones & Greenfield, 1987), leading to an enhanced passive influx of protons that can only be expelled at the expense of ATP. Alternatively or additionally, fatty-acid-limited growth may resemble other non-energy-limited conditions, such as nitrogen, phosphorus or vitamin limitation. This type of limitation may result in energy spoilage, due to uncoupling of anabolic and catabolic routes (Neijssel & Tempest, 1976*a*, *b*). On the other hand, an excess of fatty acids may also lead to uncoupling and a decreased biomass yield (Fig. 1). Micromolar amounts of octanoic and decanoic acid (provided that sufficient ethanol is present to keep them in solution) reduce the specific growth rate and yield of various yeasts, especially when the pH is lowered (Viegas *et al.*, 1989). In this case not only the effect of enhanced proton import, but also a direct effect of these medium-length fatty acids on the membrane structure has been suggested: insertion of these compounds into the membrane may decrease the hydrophobicity of the lipid portion of the membrane and increase the permeability. However, the effect of oleate will probably be less pronounced in this respect, since longer fatty acids ($>C_{10}$) are less effective in disordering membrane structure (Lee, 1976).

Table 5. *Comparison of yields, glucose uptake rates and production rates of selected metabolites during anaerobic and aerobic growth of S. cerevisiae CBS 8066 in glucose-limited chemostat cultures*

Anaerobic growth was at $D = 0{\cdot}27\ h^{-1}$ and aerobic growth was at $D = 0{\cdot}47\ h^{-1}$ (both pH 5·0). These dilution rates are close to μ_{max} (0·31 and 0·49 h^{-1}, respectively). Rates (q) are expressed as mmol (g biomass)$^{-1}$ h^{-1}.

	Anaerobic	Aerobic*
Yield (g g^{-1})	0·086	0·16
$q_{glucose}$	18·0	16·0
$q_{ethanol}$	27·7	16·5
$q_{glycerol}$	3·1	0·5
$q_{acetate}$	0·13	0·6
$q_{pyruvate}$	0·08	0·07

* Data for the aerobic culture are from Postma *et al.* (1989*a*).

Metabolic fluxes at high growth rates in aerobic and anaerobic cultures

Under aerobic conditions, *S. cerevisiae* displays the so-called Crabtree effect: occurrence of alcoholic fermentation despite oxygen excess. This leads to a physiological state which resembles that under anaerobic conditions. When an aerobic culture is shifted from a fully respiratory to an oxido-reductive metabolism, a decrease in yield is observed (Rieger *et al.*, 1983), together with an increase in the glucose uptake rate (Postma *et al.*, 1989*a*, *b*). Glucose utilization and metabolite production patterns of *S. cerevisiae* 8066 at high dilution rates under aerobic (Postma *et al.*, 1989*a*) and anaerobic conditions (this study) are similar. For instance, the glucose flux at high dilution rates is almost the same (Table 5), resulting in a comparable glycolytic flux under both conditions. Beyond the level of pyruvate, differences occur: aerobic cultures respire at the maximal rate (Barford & Hall,

1979*a*; Petrik *et al.*, 1983), and ethanolic fermentation provides the remainder of the energy required for growth. In contrast to aerobic conditions, the tricarboxylic-acid cycle has only an anabolic function during anaerobic conditions and the organism is completely dependent on ethanol formation for the provision of energy. Furthermore, under anaerobic conditions, the surplus of reducing equivalents formed in anabolic reactions must be balanced by the formation of glycerol, which in effect means that part of the glucose is lost. Some glycerol formation also occurs under aerobic conditions at high dilution rates (Postma *et al.*, 1989*a* and Table 5), indicating a redox problem.

Both aerobic cultures (at high dilution rates) as well as anaerobic cultures (at all growth rates) produced acetate. As even minute amounts of acetate formed under aerobic conditions may affect the biomass yield (Postma *et al.*, 1989*a*), the uncoupling effect of weak acids on the two yeast strains was investigated.

Effects of weak acids and culture pH

It is generally assumed that the plasma membrane of micro-organisms is only permeable to organic acids in the undissociated form, which enter by passive diffusion (Stein, 1981; Warth, 1989). Once in the cytosol, a major part of the acid will dissociate, due to the fact that the cytosolic pH (about 7) is much higher than the pK of weak acids. This results in the transfer of protons from the medium to the cytosol. In order to prevent acidification of the cytosol, this influx of protons has to be balanced via proton extrusion by the plasma-membrane ATPase, at the expense of ATP. Consequently, addition of a weak acid will result in an increased ethanol producton rate to provide the necessary ATP, and a decline in the yield. This was observed upon addition of weak acids to *S. cerevisiae* CBS 8066 and H1022 (Fig. 4). The uncoupling effect of propionate was greater than that of acetate, in line with the better liposolubility of propionic acid. A clear relationship has recently been demonstrated between the chain length of linear monocarboxylic acids and their uptake rates (Warth, 1989).

A decline in yield observed when the culture pH was lowered, without inclusion of acetate or propionate in the medium could be due to an increase in uncoupling by acetate, which is always produced in anaerobic cultures of *S. cerevisiae* CBS 8066. Since the fraction of undissociated acid will increase with decreasing pH, the degree of uncoupling is also likely to increase. However, the relationship between a low extracellular pH and uncoupling by weak acids at very low pH values appears to be rather complex. Cássio *et al.* (1987) report that *in vitro* uptake of lactate via passive diffusion by *S. cerevisiae* at different pH values was influenced by two opposing factors: an increase in uptake due to the increasing fraction of undissociated acid at lower pH, and a decrease in the diffusion rate due to decreasing membrane permeability. It follows from the data of Cássio *et al.* (1987) that at similar concentrations of undissociated lactic acid the diffusion rate across the membrane would be lower at pH 3 than at pH 5. When propionate was added to *S. cerevisiae* H1022 at pH 3·2, the decrease in cell yield in relation to the concentration of undissociated acid was considerably lower than at pH 5·0. Similar conclusions can be drawn from the data of Pampulha & Loureiro (1989) who studied the effect of acetic acid on a respiratory-deficient mutant of *S. cerevisiae* at different pH values. Furthermore, it is interesting to note that Warth (1988) reported very low intracellular concentrations of benzoate when this acid was added to chemostat cultures of various yeasts, including several *S. cerevisiae* strains, at pH 3·5. From his data an accumulation factor of only 15–20× can be calculated. The expected accumulation factor as calculated from the pK (4·19) and an estimated intracellular pH of 7·0, however, is 600×. Nevertheless, benzoate enters yeast cells in the undissociated form at a very high rate (Warth, 1989). One explanation for this remarkable observation is energy-mediated efflux of the anion, so that complete cycling is possible.

When the extracellular pH was raised to 7·1, a 20-fold increase in the acetate concentrations was observed as compared to pH 5·0 (Fig. 3). This is probably due to the rapid removal of acetic acid from the cells, at pH 7·1, so that the reaction tends towards acetate formation.

Evidently, the magnitude of uncoupling depends on a complex interplay of different variables: the cytosolic and extracellular pH, the nature and concentration of the acid, and the permeability of the plasma membrane. In view of the complexities involved, further studies regarding the effects of weak acids in yeasts are needed. In these studies the role of cell compartments such as (pro)mitochondria and vacuoles should also be included.

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