Metabolic fluxes in chemostat cultures of *Schizosaccharomyces pombe* grown on mixtures of glucose and ethanol

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Simultaneous utilization of glucose and ethanol by the yeast *Schizosaccharomyces pombe* CBS 356 was studied in aerobic chemostat cultures. In glucose-limited cultures, respirofermentative metabolism occurred at growth rates above 0.16 h^{-1} . Although *Sch. pombe* lacks a functional glyoxylate cycle and therefore cannot utilize ethanol as a sole carbon source, ethanol was co-consumed by glucose-limited chemostat cultures. As a result, biomass yields increased, but not up to the theoretical value [$0.92\text{ g biomass (g glucose)}^{-1}$] expected if all of the acetyl-CoA produced from glucose was instead synthesized from ethanol. When ethanol accounted for more than 30% of the substrate carbon in the mixed feed, it was incompletely utilized. In mixed-substrate cultures with a saturating ethanol fraction in the feed, the increase of the biomass yield as a result of ethanol consumption was highest at low dilution rates. This was not due to an increased specific rate of ethanol consumption at low growth rates; rather, the longer residence times at low dilution rates allowed *Sch. pombe* to utilize a larger fraction of the available ethanol, part of which was oxidized to acetate. Activities of gluconeogenic and glyoxylate-cycle enzymes were not detected in cell-free extracts of any of the cultures. Activities of acetaldehyde dehydrogenase and acetyl-CoA synthetase were low and of the same order of magnitude as the *in vivo* rates of acetate activation to acetyl-CoA. The results show that ethanol is a poor substrate for *Sch. pombe*, even as an auxiliary energy source.

Keywords: mixed substrates, Crabtree effect, energetics, glyoxylate cycle

INTRODUCTION

Schizosaccharomyces pombe (fission yeast) is widely used as a model organism to study the eukaryotic cell cycle. Although the physiology of *Sch. pombe* has not been studied in the same detail as that of *Saccharomyces cerevisiae* (bakers' yeast, budding yeast), it is evident that the two species share a number of key characteristics. Both are facultatively fermentative, grow on a narrow range of substrates and exhibit aerobic alcoholic fermentation in the presence of excess sugar (Fiechter *et al.*, 1981; Alexander & Jeffries, 1990).

In contrast to *Sac. cerevisiae*, *Sch. pombe* cannot use ethanol as the sole carbon source. This is probably caused by the absence of isocitrate lyase and malate synthase (Fiechter *et al.*, 1981; McDonald & Tsai, 1989). These enzymes are required for conversion of ethanol into biosynthetic intermediates containing three or more carbon atoms.

Their absence does not, however, preclude ethanol dissimilation. Indeed, it has been reported that *Sch. pombe* can dissimilate ethanol (Tsai *et al.*, 1987). Apparently, the enzymes required for conversion of ethanol into acetyl-CoA are present. This implies that *Sch. pombe* should also be able to use ethanol as a precursor for the synthesis of lipids and some amino acids.

In carbon-limited chemostat cultures, the low residual substrate concentration allows simultaneous utilization of carbon sources. This has enabled quantitative studies on the utilization of glucose/ethanol mixtures by *Sac. cerevisiae* (Geurts *et al.*, 1980; de Jong-Gubbels *et al.*, 1995). Simultaneous utilization of ethanol and glucose has also been demonstrated in chemostat cultures of *Sch. pombe* (McDonald *et al.*, 1987). However, this interesting case of mixed-substrate utilization has not been investigated quantitatively: it is unclear whether ethanol can completely replace glucose as a source of acetyl-CoA.

The aim of the present study was to determine the extent to which ethanol can contribute to the carbon and energy budget of *Sch. pombe*. To this end, growth, metabolite production and regulation of key enzyme activities were studied in aerobic chemostat cultures grown on mixtures of glucose and ethanol.

METHODS

Organism and maintenance. *Schizosaccharomyces pombe* CBS 356 was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands). Frozen stock cultures containing 20% (w/v) glycerol were stored at -70°C . Working stocks were maintained on YPD agar slants (Difco yeast extract, 10 g l^{-1} ; Difco peptone, 20 g l^{-1} ; glucose, 20 g l^{-1} ; agar, 18 g l^{-1}) at 4°C for no longer than 2 months.

Growth conditions. Carbon- and energy-limited chemostat cultivation was performed at 30°C in 2 l Applikon fermenters, at a stirrer speed of 800 r.p.m. The working volume was kept at 1.0 l by removal of effluent from the middle of the culture via an electrical level controller. The exact working volume was determined at the end of each experiment. Biomass concentrations in the effluent differed by less than 1% from those in samples taken directly from the culture. The culture pH was maintained at 5.0 by addition of 2.0 M KOH via an Applikon ADI-1020 controller. An airflow of 0.3 l min^{-1} through the cultures was maintained using a Brooks 5876 gas-flow controller. The dissolved-oxygen concentration was measured with an Ingold polarographic electrode, and, in steady-state cultures, this concentration remained constant at values above 25% air saturation. Culture purity was checked routinely by phase-contrast microscopy. The mineral medium, supplemented with vitamins and trace elements, was prepared as described by Verduyn *et al.* (1992). Glucose was sterilized separately at 110°C ; pure ethanol was added without prior sterilization. Ethanol and glucose were added at the ratios indicated, to a final concentration of 250 mM substrate carbon.

Gas analysis. The exhaust gas was cooled in a condenser (2°C) and dried with a Perma Pure dryer (PD-625-12P). O_2 and CO_2 concentrations were determined with a Servomex 1100A analyser and a Beckman model 864 infrared detector, respectively. The exhaust gas flow rate was measured as described by Weusthuis *et al.* (1994). Specific rates of CO_2 production and O_2 consumption were calculated according to the method of van Urk *et al.* (1988).

Determination of culture dry weight. Culture samples (10 ml) were filtered over preweighed nitrocellulose filters (pore size $0.45\text{ }\mu\text{m}$; Gelman Sciences). After removal of medium, the filters were washed with demineralized water, dried in a Sharp R-4700 microwave oven for 20 min at 360 W output, and weighed. Parallel samples varied by less than 1%.

Substrate and metabolite analysis. Enzymic analysis of glucose and HPLC analysis of ethanol, glycerol and organic acids were performed as described by Weusthuis *et al.* (1993). Ethanol was also determined with an enzymic assay based on alcohol oxidase (EK 001, Leeds Biochemicals). This method gave the same results, but has a lower detection limit (approximately $10\text{ }\mu\text{M}$) than the HPLC method (approximately 0.5 mM).

Preparation of cell extracts. Cells (40–80 mg dry wt) were harvested by centrifugation at 5000 g for 10 min, washed once with 100 mM potassium phosphate buffer (pH 7.5, 4°C) and resuspended in 4 ml cell disruption buffer (100 mM potassium phosphate, 2 mM MgCl_2 , 1 mM DTT, 2 mM PMSF; pH 7.5).

Cells were disrupted by sonication with 0.7 mm diameter glass beads at 0°C in an MSE sonicator (150 W output, $8\text{ }\mu\text{m}$ peak-to-peak amplitude). Six bursts of 30 s each were applied, with intermittent 30 s cooling periods. Control experiments indicated that although longer sonication resulted in an increase of the protein concentration in the extracts, it did not lead to an increase of the specific activity of glucose-6-phosphate dehydrogenase or acetyl-CoA synthetase in the cell extracts (data not shown). Whole cells and debris were removed by centrifugation at 20000 g (10 min at 4°C). The clear supernatant, typically containing 1–3 mg protein ml^{-1} , was used for enzyme assays.

Enzyme analyses. Spectrophotometric enzyme assays were performed with a Hitachi model 100-60 spectrophotometer at 30°C with freshly prepared extracts. All enzyme activities are expressed as μmol substrate converted min^{-1} (mg protein^{-1}). When necessary, extracts were diluted in sonication buffer. All assays were performed in duplicate with two concentrations of cell extract. Specific reaction rates of these duplicate experiments differed by less than 10%. In a number of cases, activities of acetyl-CoA synthetase were assayed in duplicate extracts prepared from the same culture. This resulted in specific activities which differed by less than 20%. Assays of fructose-1,6-bisphosphatase (EC 3.1.3.11), isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and pyruvate carboxylase (EC 6.4.1.1) were performed according to the method of de Jong-Gubbels *et al.* (1995). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) were assayed according to the method of Postma *et al.* (1989). Acetaldehyde dehydrogenases [NAD^{+} -dependent and NAD(P)^{+} -dependent; EC 1.2.1.4 and EC 1.2.1.5] were assayed according to the method of Postma *et al.* (1989), except that the concentration of acetaldehyde was increased to 5 mM. Acetyl-CoA synthetase (EC 6.2.1.1) was assayed according to the method of Postma *et al.* (1989), except that the concentration of potassium acetate was increased to 100 mM. In the latter assay, reaction rates in the absence of added acetate were usually negligible. However, high endogenous rates were observed in extracts from cultures which contained a high concentration of acetate. Control experiments confirmed that these extracts contained up to 1 mM acetate (data not shown). The endogenous rates were therefore assumed to represent acetyl-CoA synthetase activity and were not corrected for.

Protein determination. The protein content of whole cells was assayed by a modified biuret method (Verduyn *et al.*, 1990). Protein concentrations in cell extracts were determined by the Lowry method. Dried BSA (fatty-acid-free, Sigma) was used as a standard.

RESULTS

Growth and regulation of enzyme activities in glucose-limited chemostat cultures

In aerobic carbon-limited chemostat cultures of *Sac. cerevisiae* fed with mixtures of glucose and ethanol, the alcohol is not consumed above the critical dilution rate (D_{crit}) at which fermentation sets in (Rieger *et al.*, 1983). To choose a proper dilution rate at which to investigate mixed-substrate utilization by *Sch. pombe* CBS 356, its D_{crit} was determined and the activities of a number of key enzymes were measured in cell extracts.

In aerobic glucose-limited chemostat cultures, metab-

Fig. 1. Effect of dilution rate on the physiology of *Sch. pombe* CBS 356 in aerobic, glucose-limited chemostat cultures ($T = 30^\circ\text{C}$, pH 5.0, dissolved oxygen concentration > 25% air saturation). (a) Biomass yield on glucose (Y_{sx} , ●) and specific rate of ethanol production ($q_{ethanol}$, ○). (b) Specific rate of oxygen consumption (q_{O_2} , ●), specific rate of CO₂ production

olism was fully respiratory up to $D = 0.16\text{ h}^{-1}$ (Fig. 1a). Below 0.16 h^{-1} , the biomass yield on glucose (Y_{sx}) increased with increasing growth rate. Above 0.16 h^{-1} , ethanol was produced by the cultures and a sharp increase of the CO₂ production rate occurred (Fig. 1b). As a consequence of this onset of respirofermentative metabolism, Y_{sx} decreased sharply above D_{crit} . The specific rate of oxygen consumption (q_{O_2}) increased linearly with increasing dilution rate below D_{crit} , but decreased at the onset of respirofermentative metabolism. The maximum oxygen-consumption rate, observed at D_{crit} , was $4.4\text{ mmol O}_2\text{ g}^{-1}\text{ h}^{-1}$.

At all dilution rates, low concentrations (< 0.4 mM) of pyruvate were detected in culture supernatants. At dilution rates below 0.16 h^{-1} , glycerol concentrations were below 1 mM. Above D_{crit} , however, glycerol concentrations increased with increasing dilution rate, up to 6 mM at $D = 0.28\text{ h}^{-1}$. Acetate concentrations were below 0.2 mM at all dilution rates (data not shown).

At dilution rates below D_{crit} , a linear relationship was found between the dilution rate and the specific rate of glucose consumption ($q_{glucose}$; Fig. 1c). This allowed the estimation of the maximum biomass yield on glucose during respiratory growth ($Y_{max} = 0.51\text{ g g}^{-1}$) and of a growth-rate-independent rate of glucose consumption for maintenance ($m_s = 0.19\text{ mmol g}^{-1}\text{ h}^{-1}$; Pirt, 1965).

The key fermentative enzymes pyruvate decarboxylase and NAD⁺-dependent alcohol dehydrogenase were present in cell extracts at all dilution rates tested. Activity of alcohol dehydrogenase was essentially independent of the dilution rate up to $D = 0.28\text{ h}^{-1}$, where it decreased by approximately 50% (Fig. 2a). The activity of pyruvate decarboxylase strongly increased above the D_{crit} (Fig. 2a).

Acetaldehyde dehydrogenase and acetyl-CoA synthetase are key enzymes of ethanol metabolism, but they also contribute to glucose metabolism as part of the pyruvate dehydrogenase bypass (Holzer & Goedde, 1957; Pronk *et al.*, 1994; Flikweert *et al.*, 1996). Both NAD(P)⁺- and NAD⁺-dependent acetaldehyde dehydrogenase activities decreased substantially with increasing dilution rate, until they were barely detectable [$< 0.01\text{ U (mg protein)}^{-1}$] at $D = 0.28\text{ h}^{-1}$ (Fig. 2b). Acetyl-CoA synthetase activity did not exhibit a clear pattern. Above $D = 0.1\text{ h}^{-1}$, the activity varied between 0.05 and 0.11 U (mg protein)⁻¹. Only at the lowest dilution rate tested was a higher activity found (Fig. 2c). Activity of the anaplerotic enzyme pyruvate carboxylase appeared to decrease with increasing growth rate (Fig. 2c).

When glucose-limited chemostat cultures ($D = 0.10\text{ h}^{-1}$) were switched to a feed containing ethanol as the sole carbon source, the biomass concentration decreased

(q_{CO_2} , ○) and respiratory quotient (RQ, ■). (c) Specific rate of glucose consumption ($q_{glucose}$, ●). Carbon recoveries of the cultures were $97 \pm 1\%$. The mean protein content of the biomass was $0.40 \pm 0.03\text{ g g}^{-1}$ and did not exhibit a clear correlation with the dilution rate.

Fig. 2. Effect of dilution rate on activities of key enzymes in cell extracts of *Sch. pombe* CBS 356 grown in aerobic, glucose-limited chemostat cultures (growth conditions as in Fig. 1). (a) NAD-dependent alcohol dehydrogenase (ADH, ●) and pyruvate decarboxylase (PDC, ○); (b) NAD- (●) and NADP- (○) dependent acetaldehyde dehydrogenase; (c) acetyl-CoA synthetase (ACS, ●) and pyruvate carboxylase (PYC, ○).

Fig. 3. Utilization of glucose/ethanol mixtures by aerobic chemostat cultures of *Sch. pombe* 356 grown at a fixed D of 0.1 h⁻¹ (other growth conditions as in Fig. 1). The ethanol fraction in the feed is indicated as the fraction of the total carbon concentration (glucose+ethanol) in the medium. ●, Apparent biomass yield on glucose (Y'_{sx}); ■, specific rate of ethanol consumption ($q_{ethanol}$); □, specific rate of acetate production ($q_{acetate}$).

according to wash-out kinetics with zero growth rate (data not shown). This confirmed the previously reported inability of *Sch. pombe* to grow on ethanol as a sole carbon and energy source (Tsai *et al.*, 1987; Kotyk & Georghiou, 1994).

Mixed-substrate utilization at a fixed dilution rate

In view of the results discussed above, utilization of glucose/ethanol mixtures by *Sch. pombe* was studied at $D = 0.10$ h⁻¹. At ethanol fractions [the ethanol fraction in the feed is expressed as a fraction of the total carbon concentration (glucose+ethanol) in the medium] in the feed below 0.30 C-mol C-mol⁻¹, ethanol was completely utilized and the specific rate of ethanol consumption ($q_{ethanol}$) increased linearly with the ethanol fraction in the feed (Fig. 3). At higher ethanol fractions, a substantial part of the ethanol in the feed was not consumed, although a small further increase of $q_{ethanol}$ was observed (Fig. 3). The resulting accumulation of ethanol in the cultures was accompanied by the production of acetate (Table 1; Fig. 3). Glucose was completely consumed at all ethanol fractions tested (Table 1).

The apparent biomass yield on glucose (Y'_{sx}) increased from 0.44 g g⁻¹ in cultures grown on glucose as the sole carbon and energy source to 0.66 g g⁻¹ in cultures grown on a feed containing 0.40 C-mol ethanol C-mol⁻¹ (Fig. 3). At even higher ethanol fractions in the feed, Y'_{sx} slightly decreased, possibly as a result of uncoupling by the acetate produced by the cultures (Verduyn, 1991).

Table 1. Aerobic chemostat cultures of *Sch. pombe* CBS 356 grown on mixtures of glucose and ethanol (mean $D = 0.096 \pm 0.02 \text{ h}^{-1}$; other growth conditions as in Fig. 1)

Ethanol fraction (C-mol C-mol ⁻¹)	Reservoir concn (mM)		Residual concn (mM)		Acetate (mM)	Dry weight (g l ⁻¹)	q_{O_2} (mmol g ⁻¹ h ⁻¹)	q_{CO_2} (mmol g ⁻¹ h ⁻¹)	Carbon recovery (%)*
	Glucose	Ethanol	Glucose	Ethanol					
0	40.5	0	< 0.1	< 0.1	< 0.1	3.22	2.80	2.98	96.0
0.10	37.5	12.4	< 0.1	< 0.1	< 0.1	3.34	3.22	2.85	96.0
0.19	32.4	22.5	< 0.1	< 0.1	< 0.1	3.39	3.11	2.58	96.6
0.29	27.7	34.0	< 0.1	< 0.1	0.82	3.23	3.36	2.63	96.3
0.40	23.9	48.2	< 0.1	18.4	2.91	2.86	3.47	2.51	97.7
0.51	19.7	61.4	< 0.1	35.6	4.50	2.28	3.48	2.48	96.3
0.60	16.3	72.5	< 0.1	48.4	2.64	1.90	3.59	2.61	95.1
0.70	12.7	87.7	< 0.1	68.9	4.30	1.43	3.50	2.49	96.5

* Carbon recoveries were calculated based on a carbon content of dry biomass of 49 %.

Table 2. Enzyme activities [U (mg protein)⁻¹] in cell extracts prepared from aerobic chemostat cultures of *Sch. pombe* CBS 356 grown on mixtures of glucose and ethanol (mean $D = 0.096 \pm 0.02 \text{ h}^{-1}$; other growth conditions as in Fig. 1)

Enzyme activity	Ethanol fraction (C-mol C-mol ⁻¹)							
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Glucose-6-phosphate dehydrogenase	0.48	0.43	0.35	0.45	0.33	0.42	0.43	0.41
Alcohol dehydrogenase (NAD ⁺)	3.2	2.5	2.4	2.8	3.9	3.2	3.5	3.4
Acetaldehyde dehydrogenase (NAD ⁺)	0.17	0.17	0.11	0.06	0.09	0.05	0.06	0.06
Acetaldehyde dehydrogenase (NADP ⁺)	0.05	0.06	0.05	0.04	0.03	0.01	0.01	0.01
Acetyl-CoA synthetase	0.08	0.08	0.06	0.08	0.07	0.10	0.04	0.10

The specific rate of oxygen consumption (q_{O_2}) increased with increasing ethanol fraction in the feed (Table 1). The maximum observed oxygen-consumption rate ($3.5 \text{ mmol g}^{-1} \text{ h}^{-1}$ at an ethanol fraction of 0.70 C-mol C-mol⁻¹) was approximately 20% lower than the q_{O_2} observed at D_{crit} in aerobic chemostat cultures grown on glucose as the sole growth-limiting substrate (Fig. 1b).

To investigate the incomplete utilization of ethanol by the mixed-substrate cultures, activities of a number of key enzymes of ethanol metabolism were measured in cell extracts. Consistent with earlier reports (Fiechter *et al.*, 1981; Eraso & Gancedo, 1984), isocitrate lyase and malate synthase were not detected in extracts from cultures grown at any of the ethanol fractions tested (data not shown). The same was true for the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, although the latter enzyme can be synthesized by *Sch. pombe* (Vasarotti *et al.*, 1982; Gamo *et al.*, 1994).

Activities of glucose-6-phosphate dehydrogenase did not exhibit a clear correlation with the medium composition (Table 2). The same was true for alcohol dehydrogenase and acetyl-CoA synthetase, two key enzymes of ethanol

metabolism (Table 2). Apparently, the addition of ethanol to the medium feed did not lead to an induction of these enzymes. Peculiarly, the activities of NAD(P)⁺- and NAD⁺-dependent acetaldehyde dehydrogenases appeared to decrease with increasing ethanol concentrations in the feed (Table 2).

Effects of dilution rate on mixed-substrate utilization

In chemostat cultures, the dilution rate often has a significant impact on the utilization of mixed substrates (Rieger *et al.*, 1983; Egli *et al.*, 1986). This may involve biological mechanisms, including growth-rate-dependent expression of key enzymes in the metabolism of these substrates. However, even when levels of relevant enzymes are independent of the dilution rate, the longer residence times at low dilution rates may allow a more extensive utilization of slowly metabolizable (co-)substrates. To investigate which of these effects influence utilization of glucose/ethanol mixtures by *Sch. pombe*, chemostat cultures were grown at different dilution rates with a fixed ethanol fraction in the feed (0.60 C-mol C-mol⁻¹). This ethanol fraction was saturating, since, although glucose was completely utilized, residual ethanol

Table 3. Aerobic chemostat cultures of *Sch. pombe* CBS 356 grown at various dilution rates on a feed containing glucose and 0-60 C-mol ethanol C-mol⁻¹ (other growth conditions as in Fig. 1)

D (h ⁻¹)	Reservoir concn (mM)		Residual concn (mM)		Acetate (mM)	Dry weight (g l ⁻¹)	q_{O_2} (mmol g ⁻¹ h ⁻¹)	q_{CO_2} (mmol g ⁻¹ h ⁻¹)	Carbon recovery (%)*
	Glucose	Ethanol	Glucose	Ethanol					
0.048	16.3	77.0	< 0.1	10.5	34.1	2.20	2.57	1.46	97.9
0.075	16.3	77.0	< 0.1	32.2	21.8	2.04	2.93	2.07	97.5
0.095	16.3	72.5	< 0.1	48.7	2.6	1.90	3.59	2.61	95.1
0.121	15.9	75.0	< 0.1	55.7	3.0	1.82	3.89	2.95	95.3

* Carbon recoveries were calculated based on a carbon content of dry biomass of 49%.

Fig. 4. Effect of dilution rate on utilization of a glucose/ethanol mixture (ethanol fraction of 0.60 C-mol C-mol⁻¹) by chemostat cultures of *Sch. pombe* CBS 356. ●, Apparent biomass yield on glucose (Y_{SX}); ■, specific rate of ethanol consumption (q_{ethanol}); □, specific rate of acetate production (q_{acetate}).

was present in culture supernatants at all dilution rates tested (Table 3).

In contrast to respiratory cultures grown on glucose as

the sole carbon source (Fig. 1a), the apparent biomass yield on glucose (Y'_{SX}) in cultures grown with excess ethanol increased with decreasing dilution rate (Fig. 4). A decrease of the dilution rate from 0.12 h^{-1} to 0.05 h^{-1} led to an increase of Y'_{SX} from 0.61 to 0.74 g g^{-1} . However, the specific rate of ethanol consumption was not markedly affected by changes in the dilution rate (Fig. 4). Specific rates of acetate production increased with decreasing dilution rates, leading to acetate concentrations in the cultures of up to 34 mM at the lowest dilution rate tested (Fig. 4; Table 3). Levels of other metabolites (pyruvate, glycerol and TCA-cycle intermediates) were below 1 mM at the dilution rates tested (data not shown).

The observed growth-rate dependency of mixed-substrate utilization by *Sch. pombe* is consistent with a limited capacity of one or more key enzymes of ethanol metabolism. Since glucose was completely consumed in all cultures, attention was focused on the enzymes involved in the conversion of ethanol into acetyl-CoA, the first common intermediate in glucose and ethanol metabolism. The specific rate of ethanol consumption in the cultures grown with excess ethanol was approximately $1.5 \text{ mmol g}^{-1} \text{ h}^{-1}$ (Fig. 4). Assuming that 30% of yeast biomass consists of extractable protein (Postma *et al.*, 1989), this corresponds to a capacity of the relevant enzymes of $1.5/0.3/60 = 0.08 \text{ U (mg protein)}^{-1}$. The activities of alcohol dehydrogenase measured in extracts of the cultures grown with excess ethanol were much higher than this required value, but those of acetaldehyde

Table 4. Enzyme activities in cell extracts prepared from chemostat cultures of *Sch. pombe* CBS 356 grown at various dilution rates on a feed containing glucose and 0.60 C-mol ethanol C-mol⁻¹ (other growth conditions as in Fig. 1)

Enzyme activity	<i>D</i> (h ⁻¹)			
	0.048	0.075	0.095	0.121
Alcohol dehydrogenase (NAD ⁺)	3.4	2.9	3.5	3.2
Acetaldehyde dehydrogenase (NAD ⁺)	0.09	0.05	0.06	0.05
Acetaldehyde dehydrogenase (NADP ⁺)	0.04	0.03	0.01	0.01
Acetyl-CoA synthetase	0.04	0.08	0.08	0.11

dehydrogenases and acetyl-CoA synthetase were close to the required value (Table 4).

DISCUSSION

Aerobic fermentation in glucose-limited chemostat cultures

Sch. pombe CBS 356 exhibited a typical Crabtree-positive physiology in aerobic chemostat cultures grown on glucose as the sole carbon source, with a D_{crit} similar to that reported by Fiechter *et al.* (1981). In contrast to earlier studies (Fiechter *et al.*, 1981; Barford, 1985), a decrease of the specific oxygen-uptake rates was found above D_{crit} (Fig. 1b). It is, however, well established that the physiology of Crabtree-positive yeasts at dilution rates exceeding D_{crit} depends on culture history and in particular on the magnitude of the dilution rate shifts to which cultures have been subjected (Rieger *et al.*, 1983; Postma *et al.*, 1989; Sonnleitner & Hahnemann, 1994). The relatively large shifts in dilution rate that were applied in the present study (0.025 h^{-1}) may account for the decreasing oxygen-uptake rates at high dilution rates.

The regulation of pyruvate decarboxylase expression in *Sch. pombe* (Fig. 2a) is similar to that observed in glucose-limited chemostat cultures of *Sac. cerevisiae* (Postma *et al.*, 1989). In both yeasts, the enzyme is present at high levels during respiratory growth, and activity increases above D_{crit} . The decrease of alcohol dehydrogenase activity with increasing dilution rate found in *Sac. cerevisiae* cultures (Postma *et al.*, 1989) was not found in *Sch. pombe* (Fig. 2a). Interpretation of this difference is complicated by the presence of different isoenzymes of alcohol dehydrogenase in *Sac. cerevisiae* and *Sch. pombe* (Russell & Hall, 1983; Tsai *et al.*, 1992).

Both in *Sac. cerevisiae* (Postma *et al.*, 1989) and in *Sch. pombe* (Fig. 2b), acetaldehyde dehydrogenase activity decreased with increasing dilution rate. Thus, the capacity for conversion of acetaldehyde into acetyl-CoA decreases with increasing growth rate in both Crabtree-positive yeasts. This supports the suggestion of Postma *et al.* (1989) that a limited capacity of this pathway may be an important factor in causing aerobic fermentation. In particular, uncoupling by acetate was suggested as a possible factor in triggering aerobic fermentation. The accumulation of acetate by *Sac. cerevisiae* at high growth rates was explained by a low capacity of acetyl-CoA synthetase (Postma *et al.*, 1989). Acetate formation was not observed in aerobic cultures of *Sch. pombe* grown on glucose as the sole carbon source. This is consistent with the observation that the *in vitro* activities of acetyl-CoA synthetase in *Sch. pombe* exceeded those of acetaldehyde dehydrogenase (Fig. 2). This demonstrates that, in Crabtree-positive yeasts, aerobic alcoholic fermentation is not obligatorily linked to acetate production.

Remarkably, the low activity of pyruvate carboxylase did not increase with increasing dilution rate (Fig. 2c). Nevertheless, the *in vivo* flux through this anabolic enzyme should increase linearly with the specific growth rate. If

the required increase of the *in vivo* activity at high growth rates is achieved by an elevated intracellular pyruvate concentration, this may lead to an increase of the flux through pyruvate decarboxylase. In view of the limited capacity of the enzymes of the pyruvate dehydrogenase bypass, such a diversion of pyruvate metabolism may ultimately lead to aerobic alcoholic fermentation at high growth rates. To test whether this mechanism does indeed contribute to the occurrence of aerobic fermentation, it will be of interest to study the regulation of pyruvate carboxylase activity in other Crabtree-positive and Crabtree-negative yeasts.

Utilization of glucose/ethanol mixtures

In yeasts, a functional glyoxylate cycle is essential for the synthesis of TCA-cycle and glycolytic intermediates from C2 compounds. Because this pathway is absent in *Sch. pombe* (Eraso & Gancedo, 1984; Tsai *et al.*, 1987), ethanol can be used for two purposes only: (i) fuelling of the TCA cycle (i.e. as an auxiliary energy source) and (ii) as a source of acetyl-CoA for biosynthesis. Thus, the theoretical maximum to the Y'_{sx} in the mixed-substrate cultures can be calculated by assuming that all acetyl-CoA required for these two purposes can be derived from ethanol. These calculations are shown in Fig. 5.

The maximum Y'_{sx} observed at $D = 0.10\text{ h}^{-1}$ (0.66 g g^{-1}) in cultures grown on a feed containing $0.40\text{ C-mol ethanol C-mol}^{-1}$ (Table 1; Fig. 3) fell short of the predicted maximum value (0.92 g g^{-1} ; Fig. 5). At low dilution rates, a higher biomass yield was observed, caused by a more extensive utilization of ethanol. This was not due to physiological changes (e.g. a higher expression of ethanol-metabolizing enzymes), since the specific rate of ethanol consumption was essentially growth-rate independent (Fig. 4); rather, the long residence times at low dilution rates enable the cells to utilize more of the available ethanol. Nevertheless, even at the lowest dilution rate tested, the biomass yield remained below the theoretical value of 0.92 g g^{-1} calculated in Fig. 5.

The maximum observed oxygen-uptake rate of cultures growing on glucose/ethanol mixtures ($3.5\text{ mmol g}^{-1}\text{ h}^{-1}$; Table 1) was markedly lower than the oxygen-uptake rate of a glucose-grown culture at D_{crit} ($4.4\text{ mmol g}^{-1}\text{ h}^{-1}$; Fig. 1b). This suggests that a limitation in the synthesis of respiratory-chain components is not the reason for the incomplete utilization of ethanol; rather, the accumulation of acetate in cultures growing at ethanol fractions above $0.30\text{ C-mol C-mol}^{-1}$ (Table 1) implies a limitation in the conversion of acetate into acetyl-CoA. *Sac. cerevisiae* acetyl-CoA synthetase, the enzyme catalysing this reaction, is strongly induced when ethanol is included in the feed of glucose-limited chemostat cultures (P. de Jong-Gubbels, unpublished observations). This induction was not observed in *Sch. pombe* (Tables 2 and 4) and, at high ethanol concentrations in the feed, the enzyme activity in cell extracts was close to the calculated *in vivo* activity. Although many factors, including the intracellular concentrations of substrates and effectors, affect the flux through

Fig. 5. Schematic representation of glucose and acetyl-CoA metabolism in *Sch. pombe*. The acetyl-CoA requirement for biosynthesis [$3.4 \text{ mmol (g biomass)}^{-1}$] is based on the assumption that the macromolecular biomass composition and the amino acid composition of *Sch. pombe* are similar to values reported for *Sac. cerevisiae* (Oura, 1972; Verduyn *et al.*, 1990; Pronk *et al.*, 1994). (a) Situation in glucose-limited chemostat cultures at $D = 0.10 \text{ h}^{-1}$. The biomass yield of 0.44 g g^{-1} has been determined experimentally (see Table 1). (b) Hypothetical situation in a glucose-limited culture provided with excess ethanol, assuming that all the acetyl-CoA used in assimilation and dissimilation can be derived from ethanol. Note that *Sch. pombe* cannot use ethanol for synthesis of organic compounds with more than two carbon atoms due to the absence of a functional glyoxylate cycle.

acetyl-CoA synthetase in growing cells, the lack of induction of this enzyme by ethanol may well be the primary cause for the limited utilization of ethanol by glucose-limited cultures of *Sch. pombe*.

This study indicates that *Sch. pombe* is not well equipped for the utilization of ethanol, even when this compound is provided as a co-substrate. The absence of induction by ethanol indicates that the primary function of alcohol dehydrogenase, acetaldehyde dehydrogenase and acetyl-CoA synthetase lies in sugar metabolism, rather than in the co-consumption of ethanol. It has recently been demonstrated that the pyruvate dehydrogenase bypass is essential for growth of *Sac. cerevisiae* on glucose, probably to meet a requirement for cytosolic acetyl-CoA (Flikweert *et al.*, 1996). The constitutive expression of acetaldehyde dehydrogenase and acetyl-CoA synthetase in *Sch. pombe* implies that this pathway may play a similarly important role in this yeast.

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