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Regulation of Carbon Metabolism in Chemostat Cultures of *Saccharomyces cerevisiae* Grown on Mixtures of Glucose and Ethanol

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Growth efficiency and regulation of key enzyme activities were studied in carbon- and energy-limited chemostat cultures of *Saccharomyces cerevisiae* grown on mixtures of glucose and ethanol at a fixed dilution rate. Biomass yields on substrate carbon and oxygen could be adequately described as the net result of growth on the single substrates. Activities of isocitrate lyase and malate synthase were not detected in cell-free extracts of glucose-limited cultures. However, both enzymes were present when the ethanol fraction in the reservoir medium exceeded the theoretical minimum above which the glyoxylate cycle is required for anabolic reactions. Fructose-1,6-bisphosphatase activity was only detectable at high ethanol fractions in the feed, when activity of this enzyme was required for synthesis of hexose phosphates. Phospho-*enol*-pyruvate-carboxykinase activity was not detectable in extracts from glucose-grown cultures and increased with the ethanol fraction in the feed. It is concluded that, during carbon-limited growth of *S. cerevisiae* on mixtures of glucose and ethanol, biosynthetic intermediates with three or more carbon atoms are preferentially synthesized from glucose. Synthesis of the key enzymes of gluconeogenesis and the glyoxylate cycle is adapted to the cells' requirement for these intermediates. The gluconeogenic enzymes and their physiological antagonists (pyruvate kinase, pyruvate carboxylase and phosphofructokinase) were expressed simultaneously at high ethanol fractions in the feed. If futile cycling is prevented under these conditions, this is not primarily achieved by tight control of enzyme synthesis.

KEY WORDS — chemostat; mixed substrates; gluconeogenesis; glyoxylate cycle; *Saccharomyces cerevisiae*

INTRODUCTION

Growth of *Saccharomyces cerevisiae* in batch cultures on glucose follows a characteristic pattern. Glucose is initially mainly fermented to ethanol, which, in a separate second growth phase, serves as a carbon and energy source (Fiechter *et al.*, 1981). Adaptation to growth on ethanol involves the induction of a number of key enzyme activities, including those of the glyoxylate cycle (isocitrate lyase and malate synthase) and gluconeogenesis (phospho-*enol*-pyruvate (PEP)-carboxykinase and fructose-1,6-bisphosphatase (FBPase); Haarasilta and Oura, 1975; situations I and VI in Figure 1).

In *S. cerevisiae*, activity of the key enzymes of the glyoxylate cycle and gluconeogenesis is tightly regulated (Wills, 1990). In particular, the regulation of FBPase has been extensively studied. Activity of this enzyme is subject to a multilayered regulation, which suggests that different mechanisms have evolved to avoid the operation of a futile cycle caused by the simultaneous activity of FBPase and the glycolytic enzyme phosphofructokinase (Navas *et al.*, 1993). In *S. cerevisiae*, synthesis of FBPase is strongly repressed by glucose (Gancedo *et al.*, 1967). Furthermore, the enzyme is rapidly inactivated when glucose is added to ethanol-grown cultures (Lenz and Holzer, 1980). Catabolite inactivation by excess glucose has also been demonstrated for PEP-carboxykinase (Gancedo and Schwerzmann, 1976) and isocitrate lyase (López-Boado *et al.*, 1987). In addition to regulation of gene expression and

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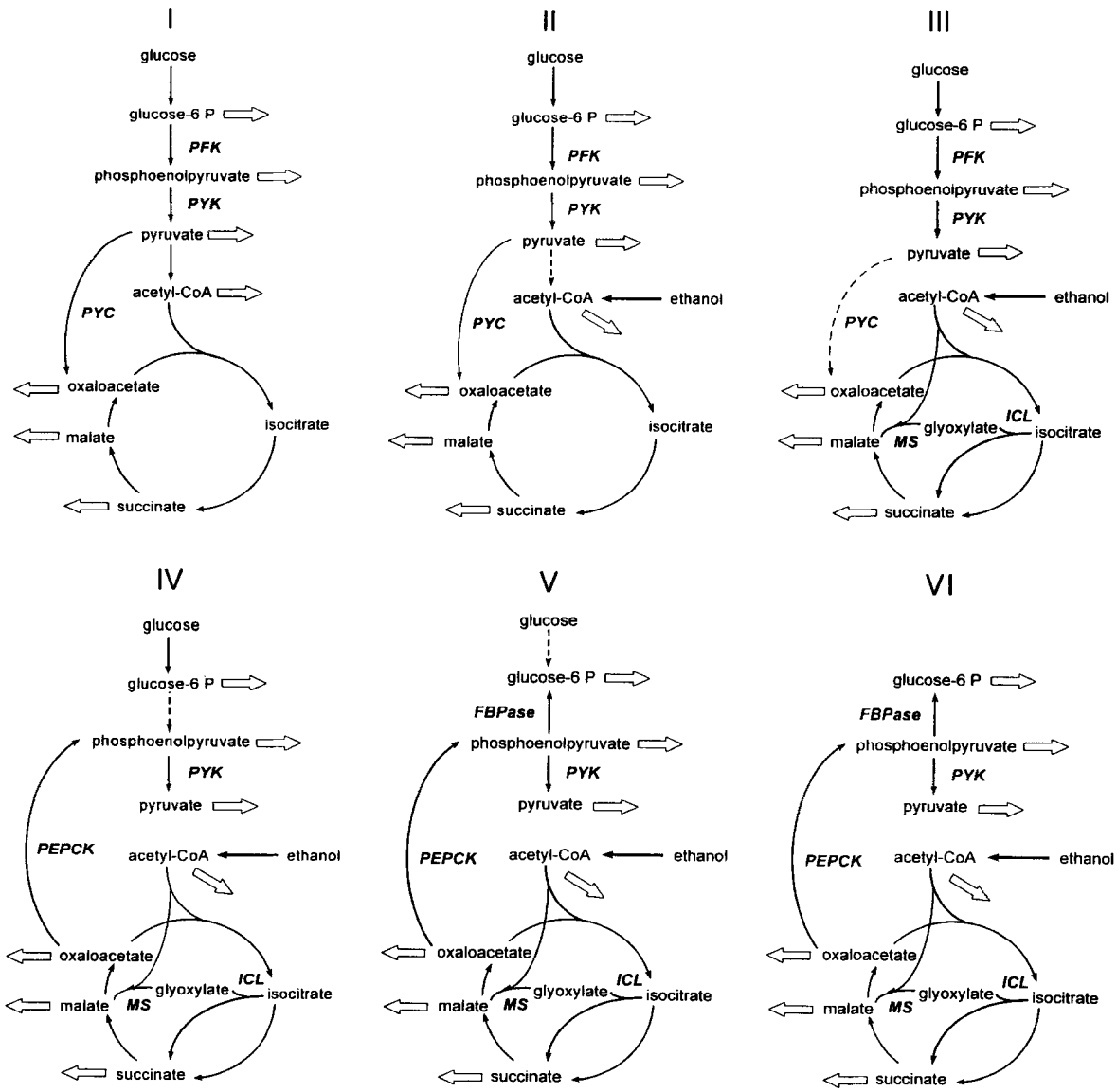

Figure 1. Schematic representation of the increasing contribution of ethanol to carbon metabolism in carbon-limited chemostat cultures of *S. cerevisiae*. Situations I and VI represent growth on the single substrates glucose and ethanol, respectively. In the intermediate situations, the ethanol fraction in the feed increases in the order II to V. At low ethanol fractions (II), ethanol is used to replace the acetyl-CoA that is derived from glucose via the pyruvate-dehydrogenase complex (dashed arrow). The limit to situation II is reached when all the acetyl-CoA used in the TCA cycle and for anabolic processes is derived from ethanol. Upon a further increase of the ethanol fraction, situation III sets in, in which synthesis of C4-compounds via the glyoxylate cycle replaces the flux via pyruvate carboxylase (dashed arrow). In situation IV, ethanol is used not only as a source of acetyl-CoA and C4-compounds, but also for synthesis of PEP. Thus, the specific feature of situation IV is the gradual replacement of glycolytic PEP synthesis (dashed arrow) by gluconeogenic synthesis via PEPCK. In situation V, even the direct phosphorylation of glucose (dashed arrow) is replaced by gluconeogenic formation of hexose phosphates, involving FBPase as a key enzyme. Open arrows indicate fluxes of intermediates that are used in anabolism. Abbreviations indicate the following key enzyme activities: PFK, phosphofructokinase; PYK, pyruvate kinase; PYC, pyruvate carboxylase; ICL, isocitrate lyase; MS, malate synthase; PEPCK, phospho-*enol*-pyruvate carboxykinase; FBPase, fructose-1,6-bisphosphatase.

catabolite inactivation, the enzymes of the glyoxylate cycle and gluconeogenesis can be regulated at the level of enzyme activity. For example, FBPase in *S. cerevisiae* is allosterically inhibited by AMP (Gancedo *et al.*, 1965) and fructose-2,6-bisphosphate (Gancedo *et al.*, 1982).

The occurrence of diauxic growth and catabolite inactivation of enzymes involved in ethanol metabolism would at first sight suggest that simultaneous utilization of ethanol and glucose does not occur in *S. cerevisiae*. However, it is well known that mixtures of substrates that lead to diauxic growth and catabolite inactivation in batch cultures can often be used simultaneously under carbon-limited growth conditions (Egli *et al.*, 1993). Indeed, it has been reported that in carbon-limited chemostat cultures, *S. cerevisiae* can simultaneously use glucose and ethanol (Geurts *et al.*, 1980). However, the regulation of key enzyme activities in such cultures has so far not been studied in detail.

When it is assumed that biosynthetic intermediates with more than two carbon atoms are preferentially synthesized from glucose, a number of situations can be envisaged for the simultaneous utilization of glucose and ethanol by *S. cerevisiae* (Figure 1). At low ethanol-to-glucose ratios, ethanol can be used to replace the acetyl-CoA that, during growth on glucose, is synthesized from pyruvate. This acetyl-CoA can be used for fuelling the TCA cycle and as a precursor for the synthesis of fatty acids and some amino acids (Figure 1, situation II). At higher ethanol concentrations, synthesis of the TCA-cycle intermediates also occurs with acetyl-CoA as a precursor. This requires the participation of the glyoxylate cycle (Figure 1, situation III). Finally, involvement of the gluconeogenic enzymes PEP-carboxykinase and FBPase allows the synthesis from ethanol of the intermediates PEP and glucose-6-phosphate (Figure 1, situations IV and V).

In the present study, the simultaneous utilization of glucose and ethanol by *S. cerevisiae* was studied in aerobic, carbon- and energy-limited chemostat cultures. The aim of this work was to investigate if the regulation of the glyoxylate-cycle and gluconeogenic enzymes in *S. cerevisiae* follows a coordinated pattern, determined by the ratio of glucose and ethanol in the feed, as suggested in Figure 1. Furthermore, the bioenergetic consequences of mixed-substrate utilization were investigated.

MATERIALS AND METHODS

Organism and maintenance

S. cerevisiae T23D (Wenzel *et al.*, 1992; Pronk *et al.*, 1994) is a homozygous diploid, homothallic wild-type strain, derived from the heterozygous strain *S. cerevisiae* CBS 8066. We are currently using *S. cerevisiae* T23D as a model organism, because its homozygous nature allows the construction of isogenic mutants. Frozen stock cultures containing 20% (w/v) glycerol were stored at −70°C. Working stocks were maintained on YEPD agar slants (Difco Yeast extract, 10 g l^{-1}; Difco peptone, 20 g l^{-1}; glucose, 20 g l^{-1}; agar, 18 g) at 4°C for no longer than 2 months.

Chemostat cultivation

Carbon- and energy-limited chemostat cultivation was performed in 2-l fermenters (Applikon, Schiedam, The Netherlands) at a dilution rate of 0·10 h^{-1}, a temperature of 30°C and a stirrer speed of 800 rpm. The culture pH was maintained at 5·0 by automatic addition of 2·0 M-KOH via an Applikon ADI-1020 controller. The working volume of the culture was kept at 1·0 l by removal of effluent from the middle of the culture via an electrical level controller. This set-up ensured that biomass concentrations in the effluent differed by less than 1% from those in samples taken directly from the culture. An airflow of 0·3 l min^{-1} through all cultures was maintained using a Brooks 5876 gas flow controller (Brooks BV, Veenendaal, The Netherlands). The dissolved-oxygen concentration was measured with an Ingold polarographic O_2-electrode and remained above 25% of air saturation. All data presented refer to carbon-limited steady-state cultures without detectable oscillations (Sonnleitner, 1991). Culture purity was checked routinely by phase-contrast microscopy at 1000 × magnification. The mineral medium contained per litre of demineralized water: $(NH_4)_2SO_4$, 5·0 g; KH_2PO_4, 3·0 g; $MgSO_4 \cdot 7H_2O$, 0·5 g; EDTA, 15·0 mg; $ZnSO_4 \cdot 7H_2O$, 4·5 mg; $CoCl_2 \cdot 6H_2O$, 0·3 mg; $MnCl_2 \cdot 4H_2O$, 1·0 mg; $CuSO_4 \cdot 5H_2O$, 0·3 mg; $CaCl_2 \cdot 2H_2O$, 4·5 mg; $FeSO_4 \cdot 7H_2O$, 3·0 mg; $NaMoO_4 \cdot 2H_2O$, 0·4 mg; H_3BO_4, 1·0 mg; KCl, 0·1 mg and silicone antifoam (BDH), 0·05 ml. After heat sterilization at 120°C and cooling, a filter-sterilized vitamin solution was added to give final concentrations of (mg l^{-1}): biotin, 0·05; calcium pantothenate, 1·0; nicotinic acid, 1·0; inositol, 25·0; thiamine · HCl, 1·0; and

para-aminobenzoic acid, 0·2. Glucose was sterilized separately at 110°C, pure ethanol was added without prior sterilization. Glucose and ethanol were added at the ratios indicated to a final concentration of 250 mM substrate carbon (0·25 CM).

Gas analysis

The exhaust gas was cooled in a condenser (2°C) and dried with a Perma Pure Dryer (PD-625-12P). O_2 consumption was determined with a Servomex 1100A Oxygen Analyser (Taylor Servomex Co., U.K.). CO_2 production by the cultures was determined with a Beckman Model 864 infrared detector. The exhaust gas flow rate was measured as described by Weusthuis *et al.* (1994). The accuracy was 3% and the repeatability 0·3%. The carbon dioxide production and the oxygen consumption rates were calculated according to van Urk *et al.* (1988).

Determination of culture dry weight

Dry weights of culture samples (10·0 ml) were determined using nitrocellulose filters (pore size 0·45 μm; Gelman Sciences, U.S.A.). After removal of the medium by filtration, the filters were washed with demineralized water and dried in an R-7400 Magnetron Oven (Sharp Inc., Japan) for 20 min. Parallel samples varied by less than 1%.

Substrate and metabolite analysis

The glucose concentrations in the reservoir media and culture supernatants were determined with the Merck glucose-oxidase kit (no. 14143). Ethanol was determined by HPLC using an HPX-87H Aminex ion-exclusion column (300 × 7·8 mm; Bio-rad) at 60°C. The column was eluted with 0·5 g l^{-1} sulphuric acid at a flow rate of 0·6 ml min^{-1}, using an ERMA ERC-7515A refractive-index detector coupled to a Hewlett Packard 3390A integrator. As an additional check, ethanol was assayed with a colorimetric assay kit (EK 003, Leeds Biochemicals, U.K.). Total-organic-carbon contents of culture supernatants were determined with a Beckman 915B TOC-analyser and corrected for the EDTA present in the trace-element solution.

Elemental composition of biomass

Samples from chemostat cultures were harvested by centrifugation, washed with demineralized water and lyophilized. Analysis of the carbon, nitrogen and hydrogen content of the lyophilized samples was performed with a Perkin-Elmer 240B elemental analyser. Unfortunately, this sample pre-treatment did not result in the complete removal of water. Therefore this method could only be used to measure C/N ratios. For the construction of carbon balances, the carbon content of dry yeast biomass was assumed to be 47·8% (J. J. Heijnen and W. van Gulik, unpublished).

Data consistency check using elemental balancing

Data were checked for consistency using the elemental balances for C, N, O and H (van der Heijden *et al.*, 1994) with the computer program Macrobal (Hellinga and Romein, 1992).

Preparation of cell-free extracts

Cells (approximately 80 mg dry wt) from steady-state cultures 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 100 mM-potassium-phosphate buffer, pH 7·5, containing 2 mM-$MgCl_2$ and 1 mM-dithiothreitol. The extracts were prepared immediately after washing by sonication of the cells with 0·7-mm diameter glass beads at 0°C for 2 min using an MSE sonicator (150 W output, 8 μm peak-to-peak amplitude). Whole cells and debris were removed by centrifugation at 20,000 *g* (10 min at 4°C). The clear supernatant, typically containing 2–4 mg protein ml^{-1}, was used as cell-free extract.

Enzyme assays

Enzyme assays were performed with a Hitachi model 100-60 spectrophotometer at 30°C with freshly prepared extracts. Reaction rates, corrected for endogenous rates, were proportional to the amount of extract added to the assays. When necessary, extracts were diluted in sonication buffer. The assays were performed at 340 nm ($E_{340\,nm}$ of reduced pyridine-dinucleotide cofactors = 6·22 mM^{-1}), except for isocitrate lyase. This enzyme was measured at a wavelength of 324 nm ($E_{324\,nm}$ of glyoxylate phenylhydrazone = 17·0 mM^{-1}). All enzyme activities are expressed as μmol of substrate converted min^{-1} per mg protein.

Fructose-1,6-bisphosphatase (EC 3.1.3.11) The reaction mixture (1 ml) contained imidazole–HCl buffer (pH 7·0), 50 μmol; KCl, 0·1 mmol; EDTA,

0·5 μmol; $MgCl_2$, 10 μmol; NADP, 0·4 μmol; phosphoglucose-isomerase (Boehringer), 0·5 U; glucose-6-P-dehydrogenase (Boehringer), 0·5 U; and cell-free extract. The reaction was started with 0·1 μmol fructose-1,6-diphosphate.

Phosphofructokinase (EC 2.7.1.11) The assay mixture (1 ml) contained imidazole–HCl (pH 7·0), 50 μmol; $(NH_4)_2SO_4$, 6·4 μmol; $MgCl_2$, 5 μmol; NADH, 0·15 μmol; ATP, 0·05 μmol; aldolase (Boehringer), 0·5 U; glycerol-P-dehydrogenase (Boehringer), 0·6 U; triose-P-isomerase (Boehringer), 1·8 U; and cell-free extract. The reaction was started with 0·25 μmol fructose-6-phosphate.

Isocitrate lyase (EC 4.1.3.1) The reaction mixture (1 ml) contained potassium-phosphate buffer (pH 7·0), 100 μmol; phenyl hydrazine, 4 μmol; cysteine, 2·5 μmol; $MgCl_2$, 2·5 μmol; and cell-free extract. The reaction was activated with 2 μmol D,L-isocitrate.

Malate synthase (EC 4.1.3.2) The assay mixture (1 ml) contained Tris–HCl buffer (pH 8·0), 0·1 mmol; acetyl-CoA, 0·4 μmol; $MgCl_2$, 5 μmol, NAD, 0·4 μmol; citrate synthase (Boehringer), 2 U; malate dehydrogenase (Boehringer), 12 U; and cell-free extract. The reaction was started with 0·25 μmol sodium glyoxylate.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) The reaction mixture (1 ml) contained imidazole–HCl buffer (pH 6·6), 100 μmol; $NaHCO_3$, 50 μmol; $MnCl_2$, 2 μmol; reduced glutathione, 2 μmol; ADP, 2·5 μmol; NADH, 0·15 μmol; malate dehydrogenase (Boehringer), 3 U; and cell-free extract. The reaction was started with 2·5 μmol PEP.

Pyruvate carboxylase (EC 6.4.1.1) The reaction mixture (1 ml) contained Tris buffer (pH 7·8), 100 μmol; $MgSO_4$, 7·5 μmol; acetyl-CoA, 0·1 μmol; $KHCO_3$, 20 μmol; NADH, 0·15 μmol; malate dehydrogenase (Boehringer), 12 U; potassium pyruvate, 10 μmol; and cell-free extract. The reaction was started with 4 μmol ATP.

Pyruvate kinase (EC 2.7.1.40) The reaction mixture (1 ml) contained cacodylate buffer (pH 6·2), 0·1 mmol; KCl, 0·1 mmol; ADP, 10 μmol; fructose-1,6-diphosphate, 1 μmol; $MgCl_2$, 25 μmol; NADH, 0·15 μmol; lactate dehydrogenase (Boehringer), 10 U; and cell-free extract. The reaction was started with 10 mM-PEP.

Protein determination

The protein content of whole cells was assayed by a modified biuret method (Verduyn *et al.*, 1990). Protein concentrations in cell-free extracts were determined by the Lowry method. In both assays, bovine serum albumin (fatty-acid free, Sigma, U.S.A.) was used as a standard.

RESULTS

Growth efficiency during simultaneous use of glucose and ethanol: theory

In the present study, growth of *S. cerevisiae* on various mixtures of glucose and ethanol was studied in carbon-limited chemostat cultures. A straightforward way to assess whether simultaneous utilization of two substrates affects growth efficiency is to first calculate the weighed sum of the respective single-substrate yields. These data can then be compared with experimentally observed yields in the mixed-substrate cultures.

When the simultaneous utilization of two substrates does not affect the overall growth efficiency, biomass yields on the mixed-substrate feed can be considered as the weighed average of the growth yields on the two individual substrates. The biomass yield on substrate carbon during growth on the mixed feed will then vary with the fraction of ethanol in the feed (f) according to equation 1.

$$Y_{SX} = f \cdot Y_{EX} + (1 - f) \cdot Y_{GX} \qquad (1)$$

In equation 1, Y_{SX} is the biomass yield on substrate carbon (g biomass [mol substrate carbon]$^{-1}$) of the mixed-substrate culture. Y_{GX} and Y_{EX} are the biomass yields on substrate carbon of glucose- and ethanol-limited chemostat cultures, respectively.

In addition to the biomass yield on substrate carbon, the biomass yield on oxygen is a key parameter. In the simple situation considered above, the overall oxygen-uptake rate by the mixed-substrate culture (qO_2) can be described as the sum of two separate contributions: one part of the culture is considered to grow on ethanol and to respire at the rate typical of ethanol-grown cells ($qO_{2,E}$). The remainder of the culture is respiring at the rate typical of glucose-grown cells ($qO_{2,G}$).

When the growth conditions are constant, the contribution of ethanol and glucose respiration then is a function of the fraction of ethanol in the feed (equation 2).

$$qO_2 = \frac{f \cdot Y_{EX} \cdot qO_{2,E} + (1-f) \cdot Y_{GX} \cdot qO_{2,G}}{f \cdot Y_{EX} + (1-f) \cdot Y_{GX}} \quad (2)$$

In chemostat cultures, the biomass yield on oxygen (Y_{OX}) is a function of qO_2 and the dilution rate (D) (equation 3).

$$Y_{OX} = \frac{D}{qO_2} \quad (3)$$

Substitution of equation 3 in equation 2 results in equation 4, which describes the relationship between the fraction of ethanol in the feed and the biomass yield on oxygen of the mixed-substrate cultures.

$$Y_{OX} = \frac{f \cdot Y_{EX} + (1-f) \cdot Y_{GX}}{\frac{f \cdot Y_{EX}}{Y_{OX,E}} + \frac{(1-f) \cdot Y_{GX}}{Y_{OX,G}}} \quad (4)$$

Deviations from the relationships between medium composition and biomass yields predicted by equations 1 and 4 should be indicative either of an increase of the growth efficiency or of a decrease of the growth efficiency (for example as a result of the occurrence of futile cycles). To investigate whether such effects occurred, biomass yields on substrate carbon and on oxygen were experimentally determined in dual-substrate-limited chemostat cultures growing at a fixed dilution rate.

Growth efficiency during simultaneous utilization of glucose and ethanol: experimental data

Chemostat cultures (D=0·10 h^{-1}) of *S. cerevisiae* T23D simultaneously utilized ethanol and glucose, indicating that complete glucose catabolite repression did not occur. The residual concentrations of ethanol and glucose in the culture supernatants were below the detection limit of the respective assays (less than 0·5 mM). A low concentration of organic carbon (typically about 4 mM) was found in supernatants of all steady-state cultures. Since no accumulation of low-molecular-weight metabolites was observed in culture supernatants by HPLC analysis, this probably reflected the presence of extracellular protein or other high-molecular-weight compounds. Carbon balances gave recoveries of 97–102%.

The biomass yields of *S. cerevisiae* in aerobic carbon-limited chemostat cultures on glucose and ethanol were comparable (15·9 and 14·0 g dry weight $Cmol^{-1}$, respectively; Figure 2) and the observed yields in the mixed-substrate cultures gave a reasonable fit with equation 1 (Figure 2). In contrast, the biomass yield on oxygen differed substantially for the two substrates. The experimental data ranged from 46·9 g dry weight (mol O_2)$^{-1}$ during growth on glucose to 16·5 g dry weight (mol O_2)$^{-1}$ during growth on ethanol as the sole carbon source (Figure 2). The growth yields on oxygen observed during growth on mixtures of glucose and ethanol were adequately described by equation 4 (Figure 2), which was based on the assumption that simultaneous utilization of glucose and ethanol can be considered as the weighed average of the growth yields on the two single substrates.

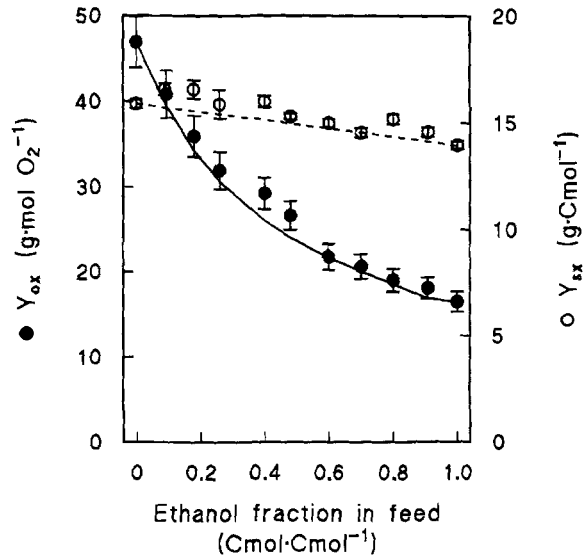

Figure 2. Effect of mixed-substrate utilization on growth efficiency of *Saccharomyces cerevisiae*. Biomass yields on substrate carbon (○) and on oxygen (●) are plotted as a function of the fraction of ethanol in the feed of aerobic, carbon-limited chemostat cultures (D=0·10 h^{-1}) grown on mixtures of glucose and ethanol. The dashed line indicates the yield on carbon substrate predicted by equation 1; the solid line indicates the yield on oxygen predicted by equation 4.

To investigate whether the composition of the yeast biomass was affected by growth on the mixed feed, the protein content and C/N ratio of biomass harvested from cultures grown at various ethanol-to-glucose ratios was determined. The protein content of the cultures was 38 ± 2·5% and did not exhibit a clear correlation with the composition of

the medium feed. Also the C/N ratio, which was 5·98 ± 0·23, did not change significantly with the composition of the reservoir medium.

Regulation of the glyoxylate cycle

During growth of *S. cerevisiae* on ethanol, synthesis of all major biopolymers (with the exception of fatty acids, which are synthesized directly from acetyl-CoA) requires the activity of the glyoxylate-cycle enzymes isocitrate lyase and malate synthase for the net synthesis of TCA-cycle intermediates. The ethanol content of the feed at which activity of these key enzymes of the glyoxylate cycle is required can be calculated by assuming that intermediates other than acetyl-CoA are preferentially synthesized from glucose (Figure 1). If this assumption is correct, activity of the glyoxylate-cycle enzymes is required when all acetyl-CoA that is dissimilated in the TCA cycle and, in addition, all acetyl-CoA that is used as a precursor for biosynthesis, is derived from ethanol.

The acetyl-CoA requirement for the formation of *S. cerevisiae* biomass is approximately 3·4 mmol g^{-1} (Oura, 1972; Pronk *et al.*, 1994). Synthesis of this amount of assimilatory acetyl-CoA from glucose requires 1·7 mmol glucose (10·2 Cmmol). Alternatively, this amount of acetyl-CoA can be synthesized from 6·8 Cmmol ethanol.

The amount of acetyl-CoA required for dissimilation can be calculated as follows. During aerobic, glucose-limited growth of *S. cerevisiae*, synthesis of 1 g biomass requires the dissimilation of approximately 3·0 mmol glucose (Pronk *et al.*, 1994). Assuming an effective P/O ratio of 1 in *S. cerevisiae* (Verduyn *et al.*, 1991), this corresponds to the generation, by substrate-level and oxidative phosphorylation, of 48 mmol ATP (Pronk *et al.*, 1994). Complete dissimilation of ethanol yields 6 moles of reduced cofactors and 1 mole of ATP equivalents from substrate-level phosphorylation in the succinyl-CoA thiolase reaction. When the effective P/O ratio for ethanol oxidation is also 1, and with an ATP requirement of 2 mol/mol acetate for the acetyl-CoA synthetase reaction (Pronk *et al.*, 1994), the net ATP yield of ethanol dissimilation is 5 mol/mol. Therefore, the 3·0 mmol glucose (18 Cmmol) that are dissimilated during glucose-limited growth can be replaced by 48 : 5 = 9·6 mmol ethanol (19·2 Cmmol).

Thus, the total amount of glucose required to synthesize the acetyl-CoA for 1 g biomass equals 10·2 + 18 = 28·2 Cmmol glucose. This amount of glucose can be replaced by 6·8 + 19·2 = 26 Cmmol ethanol. From the above and an observed glucose requirement of 62 Cmmol glucose (g biomass)$^{-1}$ (Figure 2), it follows that complete replacement of the glucose that is used for acetyl-CoA production by ethanol is predicted at an ethanol fraction of 26 : (62·9 − 28·2 + 26·0) = 0·43 Cmol Cmol^{-1}.

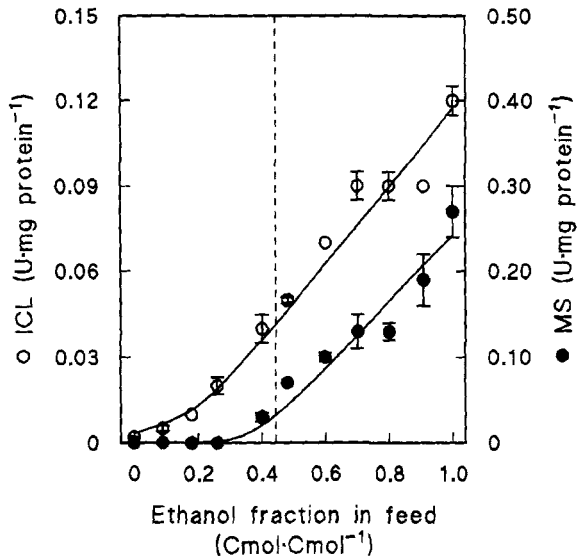

Figure 3. Regulation of glyoxylate-cycle enzymes during mixed-substrate cultivation of *S. cerevisiae* on glucose and ethanol. Activities of isocitrate-lyase (ICL, ○) and malate-synthase (MS, ●) are plotted as a function of the fraction of ethanol in the reservoir medium of aerobic, carbon-limited chemostat cultures. The dashed line indicates the ethanol fraction above which involvement of the glyoxylate cycle is theoretically required (see text). Data are presented as average ± standard deviation of two enzyme assays. Lines drawn through the data points were calculated using a smoothing algorithm (Fig. P software, Durham, U.S.A.).

In practice, the activities of isocitrate lyase and malate synthase in cell-free extracts were negligible in glucose-limited chemostat cultures (Figure 3). The activity of isocitrate lyase, however, increased substantially at low concentrations of ethanol in the feed. Malate synthase activity was only detected at ethanol fractions of 0·4 and above (Figure 3). This pattern of malate synthase activity corresponded well with the ethanol fraction at which activity of the glyoxylate cycle was theoretically predicted.

Regulation of fructose-1,6-bisphosphatase and PEP-carboxykinase

The scheme presented in Figure 1 is based on the assumption that gluconeogenic fluxes in *S.*

Table 1. Requirement of hexose phosphates for the synthesis of the major biopolymers in *Saccharomyces cerevisiae* biomass. The approximate carbohydrate, protein, nucleic acid and lipid contents of *S. cerevisiae* biomass are from Oura (1972) and Verduyn *et al.* (1990, 1991).

Polymer	g polymer (g biomass)	mmol hexose phosphate (g polymer)$^{-1}$	mmol hexose phosphate (g biomass)$^{-1}$
Carbohydrate	0·39	6·17	2·41
Protein	0·40	0·57	0·23
Nucleic acid	0·07	3·12	0·22
Lipid	0·07	—	—

Table 2. Requirement of PEP for the synthesis of the major biopolymers in *Saccharomyces cerevisiae* biomass. The approximate carbohydrate, protein, nucleic acid and lipid contents of *S. cerevisiae* biomass are from Oura (1972) and Verduyn *et al.* (1990, 1991).

Polymer	g polymer (g biomass)$^{-1}$	mmol PEP (g polymer)$^{-1}$	mmol PEP (g biomass)$^{-1}$
Carbohydrate	0·39	—	—
Protein	0·40	1·32	0·53
Nucleic acid	0·07	—	—
Lipid	0·07	—	—

cerevisiae are regulated to keep them at the minimum level required. This implies that gluconeogenesis will only occur when the amount of ethanol in the feed exceeds the amount that is required for the formation of acetyl-CoA and C4 compounds. The critical ethanol fraction in the feed above which activity of the gluconeogenic enzymes FBPase and PEP-carboxykinase is required (Figure 1, situations IV and V) can be calculated from the amounts of PEP and hexose phosphates that are required for the synthesis of biomass. To this end, we will first consider the growth of *S. cerevisiae* on ethanol. Under these conditions, both hexose phosphates and PEP are synthesized via the gluconeogenic pathway.

Approximately 2·86 mmol hexose phosphates are required for the synthesis of 1 g yeast biomass (Table 1). Formation of this amount of hexose phosphates from ethanol requires 4 moles of ethanol per mole of hexose phosphates (2 moles of carbon dioxide are lost in the PEP-carboxykinase reaction). Synthesis of the hexose phosphates in 1 g cells then requires approximately $4 \times 2{\cdot}86 = 11{\cdot}44$ mmol ethanol (22·9 Cmmol). In the ethanol-grown cultures, hexose-phosphate synthesis requires the activity of FBPase. In the dual-substrate-limited cultures, FBPase activity is no longer necessary when the glucose supply is sufficiently high to sustain the required rate of synthesis of hexose phosphates (Figure 1). This means that, per g of biomass, at least 2·86 mmol glucose (17·2 Cmmol) have to be supplied.

With a biomass yield on ethanol of 13·96 g Cmol^{-1} (Figure 2), 71·6 Cmmol ethanol is needed for the production of 1 g biomass. Replacement of the 22·9 Cmmol ethanol required for hexose-phosphate synthesis by 17·2 Cmmol glucose results in an ethanol fraction of $(71{\cdot}6 - 22{\cdot}9) : (71{\cdot}6 - 22{\cdot}9 + 17{\cdot}2) = 0{\cdot}74$ Cmol Cmol^{-1}. Theoretically, growth of *S. cerevisiae* at ethanol fractions above this critical point should require the activity of FBPase.

A similar calculation can be made to calculate the ethanol fraction at which activity of PEP-carboxykinase becomes necessary. This enzyme is required for synthesis of PEP from ethanol. PEP is an intermediate in the synthesis of some amino acids (Oura, 1972; Table 2). Synthesis of 1 g *S. cerevisiae* biomass requires 0·53 mmol PEP, which can be generated either from 0·265 mmol glucose (1·59 Cmmol) or from 1·06 mmol ethanol (2·12 Cmmol). When the PEP requirement for

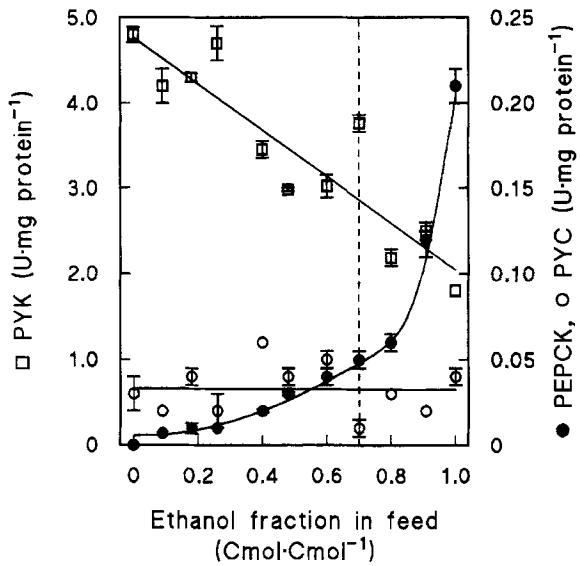

Figure 4. Regulation of PEP-carboxykinase and its physiological antagonists during mixed-substrate cultivation of *S. cerevisiae* on glucose and ethanol. Activities of PEP- carboxykinase (PEPCK, ●), pyruvate kinase (PYK, □) and pyruvate carboxylase (PYC, ○) are plotted as a function of the fraction of ethanol in the reservoir medium of aerobic, carbon-limited chemostat cultures. The dashed line indicates the ethanol fraction above which involvement of PEP-carboxykinase in biosynthetic reactions is theoretically required (see text). Data are presented as average ± standard deviation of two enzyme assays. Lines drawn through the data points were calculated using a smoothing algorithm (Fig. P. software, Durham, U.S.A.).

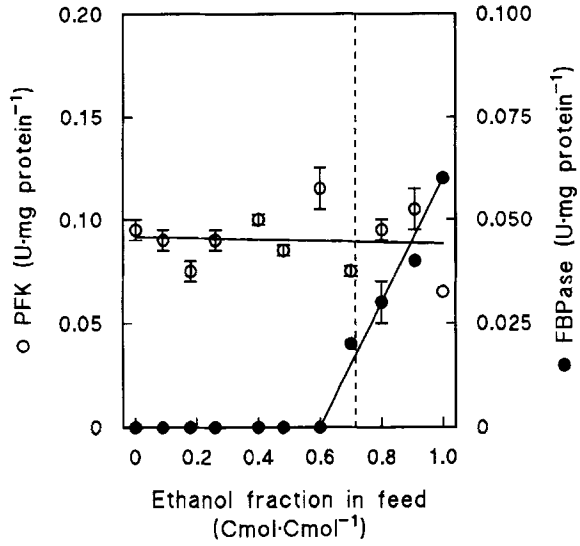

Figure 5. Regulation of FBPase (●) and its physiological antagonist phosphofructokinase (PFK, ○) during mixed-substrate cultivation of *S. cerevisiae* on glucose and ethanol. Enzyme activities are plotted as a function of the fraction of ethanol in the reservoir medium of aerobic carbon-limited chemostat cultures. The dashed line indicates that ethanol fraction above which involvement of FBPase in biosynthetic reactions is theoretically required (see text). Data are presented as average ± standard deviation of two enzyme assays. Lines drawn through the data points were calculated using a smoothing algorithm (Fig. P. software, Durham, U.S.A.).

gluconeogenic synthesis of hexose phosphates (see above) is also taken into account, the ethanol fraction at which PEP-carboxykinase activity is required corresponds to $(71{\cdot}6-22{\cdot}9-2{\cdot}12):(71{\cdot}6-22{\cdot}9-2{\cdot}12+17{\cdot}2+1{\cdot}59)=0{\cdot}71$ Cmol Cmol^{-1}.

In the dual-substrate-limited chemostat cultures, the activities of both gluconeogenic enzyme activities strongly depended on the ratio of ethanol to glucose in the feed (Figures 4 and 5). No activity of either PEP-carboxykinase or FBPase was detectable in the single-substrate, glucose-limited chemostat cultures. Addition of low concentrations of ethanol to the feed resulted in low, but significant, activities of PEP carboxykinase, which increased strongly at ethanol fractions above 0·8 (Figure 4).

In contrast to PEP-carboxykinase, FBPase activity was absent over a range of ratios of ethanol to glucose in the feed. The onset of FBPase expression occurred at an ethanol fraction of 0·7, at which the enzyme activity is theoretically required (Figure 5).

Regulation of key enzymes of glucose metabolism

The simultaneous expression of gluconeogenic enzyme activities and their glycolytic antagonists can theoretically cause futile cycles by catalysing a net hydrolysis of ATP and/or GTP. To investigate whether such a simultaneous expression occurred in the dual-substrate-limited chemostat cultures, activities of the relevant enzymes were measured in cell-free extracts.

Concerted action of the enzymes PEP-carboxykinase, pyruvate carboxylase and pyruvate kinase causes a cyclic reaction, which results in the net hydrolysis of GTP. Low but significant activities of pyruvate carboxylase were present in all chemostat cultures, even during growth on ethanol as a sole carbon source (Figure 4). Activities of the glycolytic enzyme pyruvate kinase were only approximately two-fold lower in ethanol-grown cells than in glucose-grown cells (Figure 4). Apparently, during ethanol-limited growth, the possibility of futile cycling at the level of PEP-carboxykinase is not avoided by a strict regulation of the synthesis of these two enzymes.

Another futile cycle occurs when phosphofructokinase and FBPase are simultaneously active. Phosphofructokinase was expressed constitutively and its activity did not alter significantly as a function of the ethanol fraction in the reservoir medium (Figure 5). If futile cycling at the level of FBPase is avoided in the ethanol-limited chemostat cultures, mechanisms other than regulation of enzyme synthesis must be involved.

DISCUSSION

Energetic aspects of mixed-substrate utilization

In some instances, growth of microorganisms on mixed substrates leads to an increased growth efficiency in comparison with the utilization of single substrates (Gommers *et al.*, 1988). This situation has been extensively studied in facultatively autotrophic bacteria, where mixotrophic growth results in higher yields than expected on the basis of the separate heterotrophic and autotrophic growth yields (e.g. Dijkhuizen and Harder, 1979; Gottschal and Kuenen, 1980). The biomass yields on carbon and oxygen gave a satisfactory fit with equations 1 and 4, respectively (Figure 2). Therefore, it can be concluded that utilization of mixtures of glucose and ethanol by *S. cerevisiae* did not lead to a substantial change in the growth efficiency in comparison with growth on the single substrates. A similar observation was reported by Egli *et al.* (1982a,b, 1983, 1986) for growth of *Hansenula polymorpha* and *Kloeckera* sp. on mixtures of glucose and methanol by the methylotrophic yeasts.

Regulation of metabolic fluxes during mixed-substrate utilization

The tight regulation of the key enzymes involved in ethanol assimilation, which is well documented for batch cultures (Haarasilta and Oura, 1975; Wills, 1990), was confirmed in the present study: activities of the glyoxylate-cycle and gluconeogenic enzymes were either very low or absent in cell-free extracts of glucose-limited chemostat cultures. This indicates that the absence of complete glucose repression in glucose-limited chemostat cultures (Sierkstra *et al.*, 1992) is not sufficient to trigger synthesis of these enzymes.

Although the experimental data strongly suggest that activity of the glyoxylate-cycle and gluconeogenic enzymes are regulated at the level of enzyme synthesis, the possibility that synthesis and catabolite inactivation occur simultaneously cannot be excluded. Furthermore, the actual fluxes through these enzymes are likely to be modulated by intracellular concentrations of substrates and effectors. In this respect, the compartmentation of metabolism is also an important parameter (Sulter *et al.*, 1991).

In the mixed-substrate cultures, the activity of the key enzymes of ethanol assimilation in cell-free extracts was not a simple linear function of the ethanol fraction in the feed (Figures 2–4). Furthermore, the enzymes of the glyoxylate cycle and those involved in gluconeogenesis were differentially expressed. Therefore, although common regulatory sequences have recently been identified in the structural genes encoding these enzymes (Schöler and Schüller, 1994), it is clear that gene-specific regulatory mechanisms must also be involved. The observed differential regulation of enzyme activities indicates that during mixed-substrate cultivation of *S. cerevisiae*, glucose is preferentially used as the starting material for biosynthesis and ethanol preferentially as a dissimilatory substrate (and as a source of acetyl-CoA for biosynthesis): only when the ATP requirement for glucose assimilation is completely met by oxidation of ethanol, the enzymes required for assimilation of ethanol into compounds with more than two carbon atoms are synthesized. This resembles the regulation of Calvin-cycle enzymes during mixotrophic growth of facultatively autotrophic bacteria, which are only expressed when assimilation of the organic substrate has reached its theoretical maximum (Gommers *et al.*, 1988).

The regulation of ethanol-assimilating enzymes at the level of enzyme synthesis, as observed in *S. cerevisiae*, is not representative for yeasts in general. For example, *Candida utilis* expressed substantial activities of FBPase during glucose-limited growth (Bruinenberg *et al.*, 1983) and in *Rhodotorula* yeasts, FPBase is synthesized even in the presence of excess glucose (Gancedo and Gancedo, 1971).

In contrast to the coordinated expression pattern that was observed for the key enzymes of ethanol assimilation, activities in cell-free extracts of the glycolytic enzymes phosphofructokinase and pyruvate kinase exhibited little variation with the ethanol-to-glucose ratio. This is consistent with the view that regulation of these enzyme activities is largely controlled by the concentrations of substrates and products and/or by allosteric modification.

The simultaneous expression of gluconeogenic enzymes and their physiological antagonists, observed at high ethanol fractions in the feed, can theoretically lead to futile cycling. Using ^{13}C-nuclear magnetic resonance, Navas *et al.* (1993) demonstrated that constitutive expression of gluconeogenic enzymes in a genetically engineered *S. cerevisiae* strain did not result in substantial futile cycling during growth on glucose. Apparently, low substrate pools or allosteric modification prevented *in vivo* occurrence of futile cycling. It is likely that similar mechanisms prevent *in vivo* activity of the glycolytic enzymes pyruvate kinase and phosphofructokinase during growth at high ethanol-to-glucose ratios.

In this article, the ethanol-to-glucose ratios have been calculated at which synthesis of major biosynthetic intermediates (TCA-cycle intermediates, PEP, hexose phosphates) from ethanol is a necessity for *S. cerevisiae* simultaneously utilizing the two substrates. These estimates were based on carbon fluxes only, and did not take into account other relevant factors, including cofactor specificity and ATP requirement of the reactions involved. In addition to measurements of the molecular composition of *S. cerevisiae* biomass as a function of the medium composition, a more accurate calculation of these critical points in metabolism requires the use of elaborate structured models of yeast metabolism. Such structured models are currently being developed in our laboratory (W. van Gulik *et al.*, unpublished).

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