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Anomalies in the growth kinetics of *Saccharomyces cerevisiae* strains in aerobic chemostat cultures

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Aerobic glucose-limited chemostat cultivations were conducted with *Saccharomyces cerevisiae* strains NRRL Y132, ATCC 4126 and CBS 8066, using a complex medium. At low dilution rates all three strains utilised glucose oxidatively with high biomass yield coefficients, no ethanol production and very low steady-state residual glucose concentrations in the culture. Above a threshold dilution rate, respiro-fermentative (oxido-reductive) metabolism commenced, with simultaneous respiration and fermentation occurring, which is typical of Crabtree-positive yeasts. However, at high dilution rates the three strains responded differently. At high dilution rates *S. cerevisiae* CBS 8066 produced 7–8 g ethanol L^{-1} from 20 g glucose L^{-1} with concomitant low levels of residual glucose, which increased markedly only close to the wash-out dilution rate. By contrast, in the respiro-fermentative region both *S. cerevisiae* ATCC 4126 and NRRL Y132 produced much lower levels of ethanol (3–4 g L^{-1}) than *S. cerevisiae* CBS 8066, concomitant with very high residual sugar concentrations, which was a significant deviation from Monod kinetics and appeared to be associated either with high growth rates or with a fermentative (or respiro-fermentative) metabolism. Supplementation of the cultures with inorganic or organic nutrients failed to improve ethanol production or glucose assimilation. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 231–236.

Keywords: *Saccharomyces cerevisiae*; aerobic; chemostat; growth kinetics

Introduction

Saccharomyces cerevisiae is a glucose-sensitive yeast, also termed Crabtree-positive, exhibiting aerobic ethanol production in the presence of excess glucose [2,8,21]. In aerobic chemostat culture, glucose is metabolised oxidatively at low dilution rates, resulting in a high biomass yield [11,21]. Biomass yield coefficients ranging from 0.47 to 0.50 g g glucose^{-1} have been reported for different *S. cerevisiae* strains [13,14]. At dilution rates above a critical value, metabolism of the yeast shifts towards a respiro-fermentative (also termed oxido-reductive) type of metabolism, with aerobic ethanol production [11].

Above this critical dilution rate, an ethanol yield of 0.32 g g glucose^{-1} was reported for *S. uvarum* [11] and 0.34 g g^{-1} for *S. cerevisiae* H1022 [14]. This respiro-fermentative region of a chemostat culture is also characterised by a decrease in the biomass yield to values ranging from 0.1 [11,13] to 0.21 g g glucose^{-1} [14]. Steady-state residual glucose concentrations in the culture typically remain low until the wash-out dilution rate is approached [13,20].

We found, however, that in aerobic cultures of *S. cerevisiae* strains ATCC 4126 and NRRL Y132 unusually high steady-state residual sugar levels occurred in the respiro-fermentative region. This was a significant deviation from Monod kinetics, considering that the culture was clearly glucose-limited when grown at dilution rates in the respiratory region. A similar trend may be seen in the data for *S. cerevisiae* CEN.PK113–7D grown in an aerobic glucose-limited chemostat culture [5], suggesting that this phenomenon may also be found in various other strains of *S. cerevisiae*.

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Materials and methods

Organisms and maintenance

Saccharomyces cerevisiae strains NRRL Y132, ATCC 4126 (from the culture collection of the Dept of Microbiology and Biochemistry, UOFS, Bloemfontein, South Africa) and *S. cerevisiae* CBS 8066 (Centraalbureau voor Schimmelcultures, Delft, the Netherlands) were maintained on Difco Yeast Nitrogen Base agar slants at 4°C.

Chemostat cultivation

Experiments involving continuous culture were conducted in a 2-litre stirred tank reactor with a working volume of 700–800 ml (Multigen F-2000; New Brunswick Scientific, Edison, NJ, USA), fitted with an exhaust gas condenser, polarographic oxygen electrode (Ingold AG, Urdorf, Switzerland) and a pH electrode (Mettler Toledo, Halstead, UK) at 30°C and pH 5.5. Silicone rubber tubing with peristaltic pumps (Watson Marlow, Cornwall, UK) was used for the medium feed and level control by withdrawal of excess culture from the culture surface. The dissolved oxygen tension (DOT) was regulated at or above 20% of air saturation by maintaining an aeration rate of 1 L min^{-1} and manually adjusting the stirrer speed of the bioreactor within the range of 400–1000 rpm. The complex medium comprised (per litre distilled water): glucose, 20 g; $(NH_4)_2SO_4$, 5.5 g; KH_2PO_4, 3.4 g; $MgSO_4 \cdot 7H_2O$, 0.8 g; $CaCl_2 \cdot 2H_2O$,

0.05 g; citric acid, 0.25 g; yeast extract (Biolab Diagnostics, Midrand, South Africa), 3.0 g and trace elements as reported elsewhere [7]. Silfoamex CF antifoam (SA Silicones, Boksburg, South Africa) or Dow Corning 1520 Silicone antifoam (Dow Corning, Seneffe, Belgium) was added at 0.75 ml per litre to suppress foaming. Variations of the above complex medium were used in some experiments, namely: (a) using double distilled water instead of single distilled water; (b) without the addition of trace elements, also using double distilled water; and (c) without citric acid.

Samples were taken at steady state, usually after three to five residence times, except when shifts from the respiratory region to the respiro-fermentative region were carried out, in which case at least eight residence times were allowed before sampling. Steady state was defined as a variation of less than 7% in the culture turbidity for at least three consecutive residence times with no upward or downward trend. In some experiments exhaust gas analysis was also used to verify steady state conditions.

The critical wash-out dilution rate, numerically equivalent to the maximum specific growth rate (μ_{max}), was determined by partially washing out a culture adapted to a high growth rate and measuring the growth rate after interruption of the medium feed. The specific rates of ethanol production (q_p) were calculated according to $q_p = D.p/x$, where p and x are the ethanol and biomass concentrations, respectively, at steady state. The specific rate of glucose assimilation (q_s) was calculated as $q_s = D\ (s_r - s)/x$, where s is the steady-state residual glucose concentration and s_r the glucose concentration in the sterile medium reservoir.

Shift and pulse experiments

The effect of a nutrient pulse was investigated by addition of the following nutrients dissolved in 20 ml distilled water directly into the culture at steady-state in the respiro-fermentative region: (a) $(NH_4)_2SO_4$, 4.4 g and yeast extract, 2.4 g; (b) KH_2PO_4, 2.72 g and $MgSO_4{\cdot}7H_2O$, 0.64 g; (c) trace elements ($FeSO_4{\cdot}7H_2O$, 0.028 g; $MnSO_4{\cdot}H_2O$, 0.0056 g; $ZnSO_4{\cdot}7H_2O$, 0.0088 g; $CuSO_4{\cdot}5H_2O$, 0.0008 g; $CoCl_2{\cdot}6H_2O$, 0.0016 g; $NaMoO_4{\cdot}2H_2O$, 0.00104 g; KI, 0.0028 g; H_3BO_3, 0.0016 g; $Al_2(SO_4)_3{\cdot}18H_2O$, 0.00128 g) and $CaCl_2{\cdot}2H_2O$, 0.04 g.

Analytical procedures

Periodic samples (20 ml) were taken aseptically and kept on ice until analyzed. Growth was monitored with a Klett-Summerson colorimeter (Klett Mfg Co, New York, USA) fitted with a red filter (No. 66). The cell concentration was gravimetically determined in duplicate by drying centrifuged and washed samples at 105°C. The glucose content of the culture supernatants was determined with a Sugar Analyser I high-performance liquid chromatograph equipped with a refractive index detector and a Sugarpack I column (Waters Associates, Milford, MA, USA) operating at 85°C with an eluent (degassed water) flow rate of 0.5 ml min^{-1}. Glucose concentrations below 1 g L^{-1} were analysed with a D-glucose enzymatic bioanalysis kit (Cat. No. 716251, Boehringer Mannheim, Mannheim, Germany). For these enzymatic assays, rapid harvesting of the cells was essential; therefore, biological activity in the samples was rapidly inactivated by aspirating them into 30-ml sample bottles, each containing 500 μl 5 N HCl, and immediately put on ice. Ethanol concentrations were determined with a gas chromatograph (model 5710A; Hewlett-Packard, Atlanta, GA, USA) equipped with a glass column (1.5 m × 1.5 mm ID), packed with 80–100 mesh Porapak N (Waters Associates) and with 50 ml nitrogen carrier gas min^{-1} at an oven temperature of 165°C. The composition of the exhaust gas was determined using an infrared Uras 10E CO_2 analyser and a paramagnetic Magnos 6G O_2 analyser (Hartmann & Braun AG, Frankfurt, Germany). The rates of gas exchange were calculated by means of a nitrogen balance.

Results and discussion

In agreement with previous reports [3,9], at low dilution rates respiratory growth occurred with a high biomass yield, no or very little ethanol production (<80 mg L^{-1}) and very little residual glucose in aerobic glucose-limited steady-state cultures of *S. cerevisiae* NRRL Y132 (data not shown), ATCC 4126 and CBS 8066 (Figures 1 and 2,

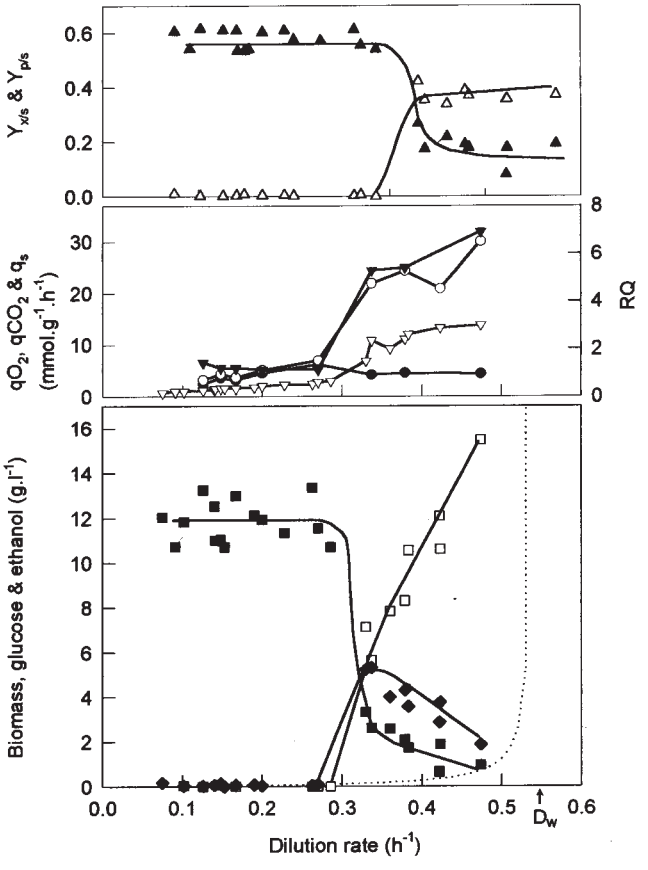

Figure 1 Steady-state concentrations of biomass (■), glucose (□) and ethanol (◆) in aerobic glucose-limited chemostat cultures of *S. cerevisiae* ATCC 4126 in a complex medium containing 20 g glucose L^{-1}. The respiratory quotient (RQ, ▼), the yield coefficients for biomass (▲) and ethanol (△), as well as the specific rates of glucose (q_s, ▽) and oxygen uptake (qO_2, ●) and CO_2 evolution (qCO_2, ○) are indicated. The broken line indicates the residual glucose values predicted by the Monod equation, using an arbitrary k_s value of 100 mg glucose L^{-1}. The wash-out dilution rate (D_w) is indicated by an arrow. The data points are from eight independent experiments.

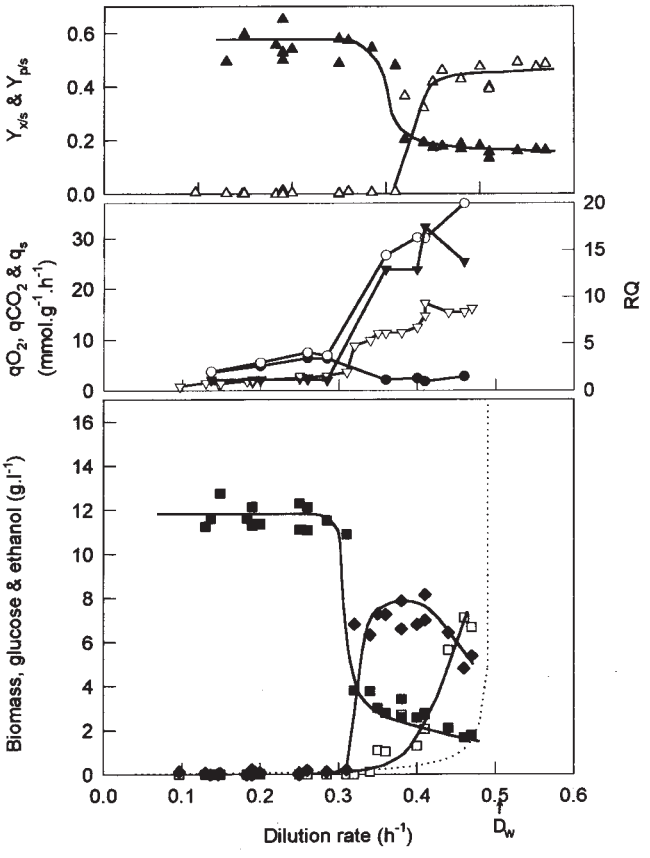

Figure 2 Steady-state concentrations of biomass (■), glucose (□) and ethanol (◆) in aerobic glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 in a complex medium containing 20 g glucose L^{-1}. The respiratory quotient (RQ, ▼), the yield coefficients for biomass (▲) and ethanol (△), as well as the specific rates of glucose (q_s, ▽) and oxygen uptake (qO_2, ●) and CO_2 evolution (qCO_2, ○) are indicated. The broken line indicates residual glucose values predicted by the Monod equation, using an arbitrary k_s value of 100 mg glucose L^{-1}. The wash-out dilution rate (D_w) is indicated by an arrow. Data points are from seven independent experiments.

Table 1). The biomass yield coefficients of the strains were slightly higher than the theoretical maximum of 0.511 g g glucose^{-1} [19], probably because yeast extract in the medium also served as carbon source for biomass formation. The cell concentration in the outflow from the vessel was identical to that of the culture in the vessel, indicating homogeneous culture removal. The scatter in the biomass data points resulted mainly from variations in the glucose content of the many batches of medium used.

At a critical dilution rate, the limited respiratory capacity of the cells apparently became saturated [17] and with a further increase in dilution rate the metabolism of the yeasts shifted towards a respiro-fermentative (oxido-reductive) metabolism with aerobic ethanol production taking place. This critical dilution rate, up to where the metabolism was completely oxidative, namely 0.29 h^{-1}, was similar for these yeast strains (Table 1). These values were, therefore, between 50% and 65% of the μ_{max} values of these strains, as determined by wash-out experiments. A previous report indicated that the critical dilution rate of *S. cerevisiae* CBS 8066 was 0.38 h^{-1} [13]. Due to ethanol production, the respective biomass yield coefficients decreased to between

Table 1 Kinetic and stoichiometric parameters of *S. cerevisiae* strains grown in aerobic glucose-limited chemostat cultures at different dilution rates in a complex medium

Parameter	*S. cerevisiae* ATCC 4126	*S. cerevisiae* NRRL Y132	*S. cerevisiae* CBS 8066
Respiratory region			
D (h^{-1})	0.15	0.14	0.19
$Y_{x/s}$ (g g glucose^{-1})	0.54	0.52	0.5
$Y_{p/s}$ (g g glucose^{-1})	0	0	0
q_s (g g^{-1} h^{-1})	0.28	0.27	0.38
q_p (g g^{-1} h^{-1})	0	0	0.044
E (%)	100	100	100
Respiro-fermentative region			
D (h^{-1})	0.38	0.44	0.41
$Y_{x/s}$ (g g glucose^{-1})	0.18	0.1	0.13
$Y_{p/s}$ (g g glucose^{-1})	0.37	0.33	0.39
q_s (g g^{-1} h^{-1})	2.15	4.59	3.09
q_p (g g^{-1} h^{-1})	0.79	1.54	1.21
E (%)	47.81	36.62	98.77
k_s (mg L^{-1})	60	ND	40
D_c (h^{-1})	0.29	0.29	0.29
μ_{max} (h^{-1})	0.54	0.45	0.5

D, Dilution rate.
D_c, Critical dilution rate; the highest D value where the metabolism was completely oxidative.
E (%), Efficiency of glucose utilisation, (g glucose assimilated g^{-1} glucose supplied) ×100.
k_s, Saturation constant in Monod equation.
q_s, Specific rate of substrate assimilation.
q_p, Specific rate of ethanol production.
$Y_{x/s}$, Biomass yield coefficient.
$Y_{p/s}$, Ethanol yield coefficient.
μ_{max}, Maximum specific growth rate.
ND, Not determined.

0.1 and 0.18 with ethanol yield coefficients of 0.33–0.39 (Table 1). However, in the respiro-fermentative region of cultures of strains NRRL Y132 and ATCC 4126, up to 10 g glucose L^{-1} (*ca* 50% of the glucose concentration in the feed) remained unutilised at steady state, resulting in production of only 3–4 g ethanol L^{-1}. In anoxic batch cultures both of these strains produced up to 83 g ethanol L^{-1} from 200 g glucose L^{-1} (data not shown); an abnormally low ethanol tolerance could, therefore, not be the explanation for this observation.

The steady-state residual glucose values predicted for cultures of *S. cerevisiae* ATCC 4126 and CBS 8066 by a transformation of the Monod model, $s = k_s[D/(\mu_{max} - D)]$, using an arbitrary k_s value of 100 mg L^{-1} [10] and the μ_{max} values as determined by wash-out experiments (Table 1), are indicated by broken lines in Figures 1 and 2. Although with strain CBS 8066 the steady-state residual glucose concentrations at high dilution rates were higher than predicted, this was in sharp contrast to the very high residual glucose concentrations noted in cultures of the other two strains. Due to the sharp increase in residual glucose, non-linear regression using the Monod model could not be used to determine the k_s values. The residual glucose concentration at a dilution rate equivalent to 0.5 μ_{max} was, therefore, used as an estimate of the k_s value (Table 1).

Trends in the specific rates of oxygen consumption (qO_2) and CO_2 production (qCO_2) by *S. cerevisiae* ATCC 4126 and CBS 8066 as a function of dilution rate were similar,

but the respiratory quotient (RQ) value of strain CBS 8066 in the respiro-fermentative region was about double that of strain ATCC 4126 (Figures 1 and 2). The qO_2 and qCO_2 values of both strains gradually increased with an increase in dilution rate up to the critical dilution rate, with a respiratory quotient of just above one (1.19 ± 0.18), indicating respiratory metabolism. Above the critical dilution rate the qCO_2 value increased sharply, resulting in a high RQ value which coincided with the formation of ethanol. The slight decrease in the qO_2 values above the critical dilution rate might have been due to insufficient adaptation or due to the presence of ethanol. It has been shown that the 'respiratory repression' at high dilution rates could be eliminated after sufficient adaptation, which required about 50 generations [1]. It was also shown that ethanol exerted a negative effect on respiration and could possibly suppress the upper limit of respiration below the maximal value [16].

Oscillations in the gas exchange values were observed at low dilution rates. Synchronised metabolic oscillations in aerobic chemostat cultures is a well-known phenomenon [12,15,22]. As observed previously, the oscillatory growth originated spontaneously after the chemostat was started and it was maintained for as long as the chemostat was operated [12,18]. This oscillatory behaviour was observed between dilution rates of 0.126 h^{-1} and 0.2 h^{-1} with strain ATCC 4126 and between 0.135 h^{-1} and 0.285 h^{-1} with strain CBS 8066 (data not shown). An increase in the dilution rate of the ATCC 4126 culture resulted in a longer oscillatory cycle time. A similar phenomenon was also observed with strain CBS 8066, but only up to a dilution rate of 0.26 h^{-1}, at a dilution rate of 0.285 h^{-1} the oscillatory period decreased again.

Oscillations in the dissolved oxygen tension (DOT) and ethanol concentration correlated with oscillations in the O_2 and CO_2 content of the exhaust gas (Figure 3). These oscillations, therefore, exerted a slight but significant effect on the steady-state values reported in this paper. Many cyclic parameter changes have been reported during oscillatory growth [6,15]. A 10% change in biomass concentration at a dilution rate of 0.07 h^{-1} [22] and a variation of about 25% in the biomass yield at a dilution rate of 0.1 h^{-1} [6] during oscillatory growth have been reported. By contrast, others found no variation in the biomass concentration under similar conditions [15].

The physiological difference between strains CBS 8066 and ATCC 4126 also was evident from the transients during dilution rate shift experiments. A steady-state culture of *S. cerevisiae* CBS 8066 at a dilution rate of 0.19 h^{-1} (in the respiratory region) was subjected to a one-step shift-up to a dilution rate of 0.38 h^{-1} (in the respiro-fermentative region) (Figure 4). The immediate decrease in biomass concentration, concomitant with an increase in ethanol concentration, was in agreement with previous shift experiments done with *Saccharomyces uvarum* H2055 [11]. However, whereas in our experiments the glucose concentration increased after about 7 h and again decreased to undetectably low concentrations 8 h later, an almost immediate increase in glucose concentration was observed with *S. uvarum* H2055 after a shift from a dilution rate from 0.14 h^{-1} to 0.21 h^{-1}, followed by a decrease after about 6 min [11].

The response of *S. cerevisiae* ATCC 4126 in a similar

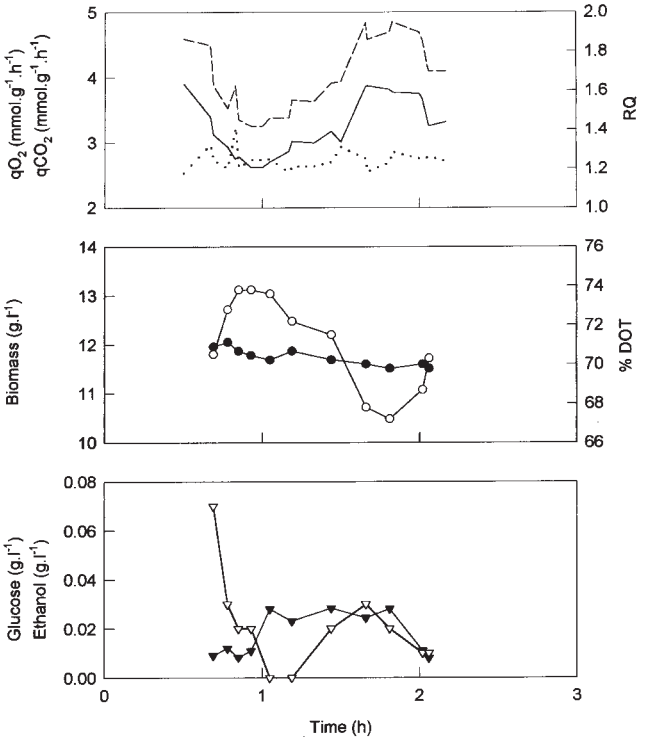

Figure 3 The concentrations of biomass (●), glucose (▼) and ethanol (▽), dissolved oxygen tension (DOT, ○) and RQ values (· · ·) and the content of O_2 (– – –) and CO_2 (——) in the exhaust gas over time in an aerobic, glucose-limited chemostat culture of *S. cerevisiae* ATCC 4126 at a dilution rate of 0.167 h^{-1}.

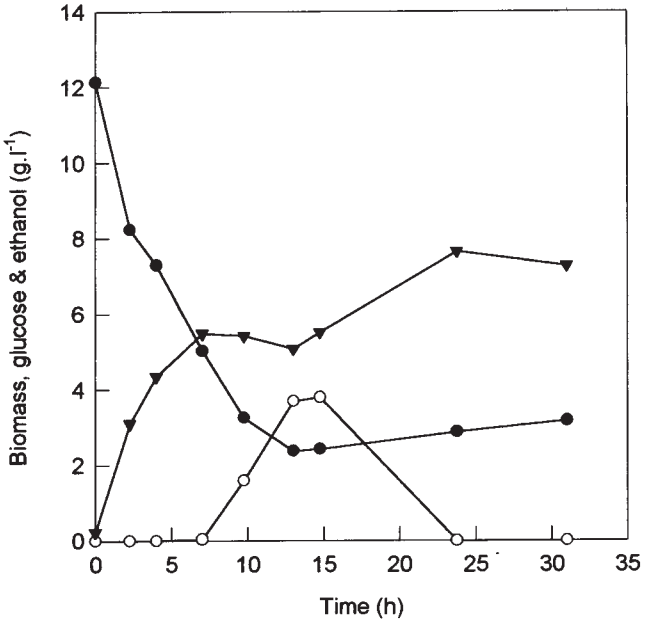

Figure 4 The effect of a dilution rate shift (at time zero) on an aerobic chemostat culture of *S. cerevisiae* CBS 8066 from an initial dilution rate of 0.19 h^{-1} to 0.38 h^{-1}. The values at time zero are the steady-state values at a dilution rate of 0.19 h^{-1}. Symbols: biomass (●), glucose (○) and ethanol (▼).

experiment was quite different (Figure 5). The dilution rate of a steady state culture was increased from 0.14 h^{-1} to 0.361 h^{-1}. However, a slight lag in ethanol production was evident. The glucose concentration increased after about 6 h, but in this instance it stabilised at about 8 g L^{-1}, resulting in the production of only 3.5 g ethanol L^{-1}.

To establish whether the above aberrant chemostat behaviour might have been due to an unknown nutrient limitation, several pulse additions of various nutrients (see Materials and Methods) were made directly into a steady-state culture of *S. cerevisiae* ATCC 4126 in the respiro-fermentative region (data not shown). None of the pulses elicited either an increase in the ethanol concentration or a decrease in the high residual sugar concentration. Increasing the glucose concentration in the feed resulted in an increased biomass concentration with both the ATCC 4126 (Figure 6) and the CBS 8066 (data not shown) strains, confirming that the chemostat was operating under a glucose limitation. Further experiments with variations of the medium normally used (see Materials and Methods) did not result in any significant improvement in ethanol production or sugar assimilation (data not shown). In fact, the residual glucose concentration decreased only slightly. These results confirmed that the culture was not limited in respect to nitrogen, minerals or growth factors (as supplied by the pulse of yeast extract), nor were high residual glucose levels due to inhibitory effects of citric acid (used as chelating agent) or the trace elements added.

A similar increase in the steady-state residual glucose concentration at dilution rates above 0.18 h^{-1} was found with both the ATCC 4126 and CBS 8066 strains in anoxic chemostat cultures using the same complex medium [4]. Thus, the above aberrant behaviour occurred under both anoxic and aerobic conditions, and appeared to be associated either with high growth rates or with a fermentative (or respiro-fermentative) metabolism. These results show that distinct differences in the kinetic behaviour exist between strains of the same yeast species, which may impact on the industrial applications of *S. cerevisiae*. This deviation in Monod kinetics is obviously of importance in studies of the kinetics or physiology of *S. cerevisiae* in carbon-limited continuous culture. Further experiments are being conducted to determine whether this phenomenon was due to nutrient limitations other than those tested here by pulse experiments.

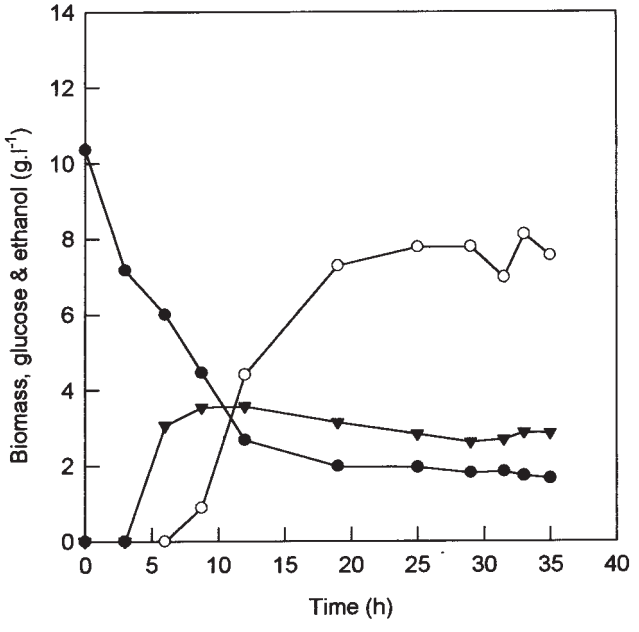

Figure 5 The effect of a dilution rate shift (at time zero) on an aerobic chemostat culture of *S. cerevisiae* ATCC 4126 from an initial dilution rate of 0.14 h^{-1} to 0.361 h^{-1}. The values at time zero are steady-state values at a dilution rate of 0.14 h^{-1}. Symbols: biomass (●), glucose (○) and ethanol (▼).

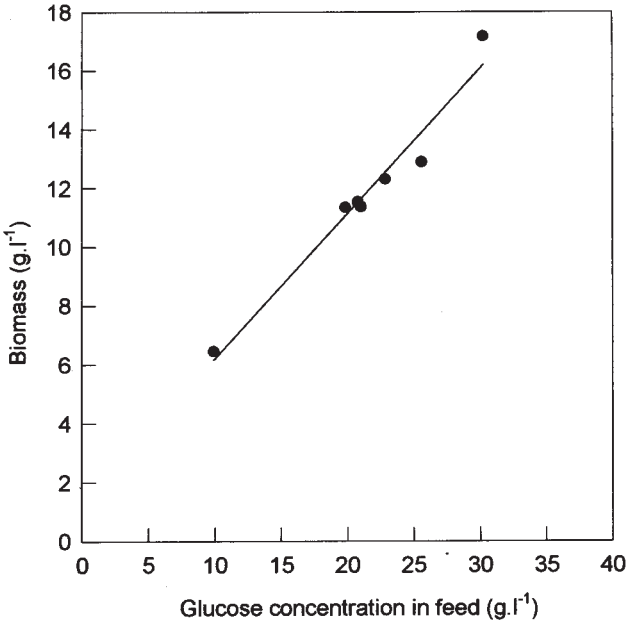

Figure 6 The steady-state biomass concentration in an aerobic chemostat culture of *S. cerevisiae* ATCC 4126 as a function of the glucose concentration in the feed at a dilution rate of 0.25 (±0.02) h^{-1}.

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