

## Quantitative Physiology of *Saccharomyces cerevisiae* at Near-Zero Specific Growth Rates<sup>∇</sup>

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**Growth at near-zero specific growth rates is a largely unexplored area of yeast physiology. To investigate the physiology of *Saccharomyces cerevisiae* under these conditions, the effluent removal pipe of anaerobic, glucose-limited chemostat culture (dilution rate, 0.025 h<sup>-1</sup>) was fitted with a 0.22- $\mu$ m-pore-size polypropylene filter unit. This setup enabled prolonged cultivation with complete cell retention. After 22 days of cultivation, specific growth rates had decreased below 0.001 h<sup>-1</sup> (doubling time of >700 h). Over this period, viability of the retentostat cultures decreased to ca. 80%. The viable biomass concentration in the retentostats could be accurately predicted by a maintenance coefficient of 0.50 mmol of glucose g<sup>-1</sup> of biomass h<sup>-1</sup> calculated from anaerobic, glucose-limited chemostat cultures grown at dilution rates of 0.025 to 0.20 h<sup>-1</sup>. This indicated that, in contrast to the situation in several prokaryotes, maintenance energy requirements in *S. cerevisiae* do not substantially change at near-zero specific growth rates. After 22 days of retentostat cultivation, glucose metabolism was predominantly geared toward alcoholic fermentation to meet maintenance energy requirements. The strict correlation between glycerol production and biomass formation observed at higher specific growth rates was not maintained at the near-zero growth rates reached in the retentostat cultures. In addition to glycerol, the organic acids acetate, D-lactate, and succinate were produced at low rates during prolonged retentostat cultivation. This study identifies robustness and by-product formation as key issues in attempts to uncouple growth and product formation in *S. cerevisiae*.**

Laboratory studies on microorganisms are often performed in batch cultures. During the initial phase of batch cultivation, all nutrients are usually present in excess. As a consequence, the initial specific growth rate,  $\mu$ , of the microorganism in such cultures equals the maximum specific growth rate,  $\mu_{\max}$ . In natural environments, the specific growth rate of microorganisms is likely to be constrained by the limited availability of one or more growth-limiting nutrients, resulting in specific growth rates far below  $\mu_{\max}$  (8, 24). In chemostat cultures fed with a medium containing a single growth-limiting nutrient, the dilution rate determines the specific growth rate. Chemostat cultivation therefore offers the possibility to study microbial physiology at carefully controlled, submaximal specific growth rates and to investigate the effect of specific growth rate on cellular physiology (20). Chemostat cultivation of the yeast *Saccharomyces cerevisiae* has demonstrated strong effects of specific growth rate on biomass composition (26, 51), product formation (5, 37), and cell size (23). Moreover, during energy-limited growth at low specific growth rates, a relatively large fraction of the energy substrate has to be dissipated for maintenance-related processes such as maintenance of chemi-osmotic gradients and turnover of cellular components (34). Not surprisingly, recent genome-wide studies have shown strong effects of specific growth rate on levels of mRNAs and proteins (9, 14, 38).

In chemostat studies on *S. cerevisiae*, the steady-state specific growth rate is usually between 0.03 h<sup>-1</sup> and 0.40 h<sup>-1</sup>. While this range is relevant for many industrial applications, there are several incentives to study growth of this yeast at even lower specific growth rates. In many natural environments, growth at a  $\mu$  of 0.03 h<sup>-1</sup>, corresponding to a doubling time of 23.1 h, probably still represents extremely fast growth. Furthermore, in industrial applications, *S. cerevisiae* and other microorganisms can be considered as self-replicating catalysts, and, unless biomass is the desired product, growth can be considered as undesirable by-product formation leading to nonproductive substrate consumption. This problem is further augmented when the excess yeast biomass cannot be valorized because it is genetically modified or has been used for the production of compounds that are not compatible with use as, for example, cattle feed. A third incentive for exploring the physiology of *S. cerevisiae* at near-zero growth rates is related to the increasing interest in this yeast as a systems biology model for human cells (16, 27, 33). At near-zero growth rates, the age of individual yeast cells becomes much higher than can be achieved in conventional batch or chemostat cultures. Studies on extremely slow growth of *S. cerevisiae* under defined conditions may therefore provide an interesting model for ageing of human cells.

Retentostat cultivation, first proposed by Herbert (18), is a modification of chemostat cultivation that has been specifically designed to study microbial physiology at near-zero specific growth rates. In a retentostat, sometimes referred to as recycling fermentor or recyclost, the growth-limiting energy substrate is fed at a constant rate, and biomass is retained in the

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fermentor by an internal filter probe connected to the effluent line or by an external filter module. Prolonged retentostat cultivation should, in theory, result in a situation where the specific growth rate becomes zero and where the specific rate of substrate consumption equals the maintenance energy requirement. This situation is fundamentally different from starvation, which involves deterioration of physiological processes, and from resting states typified by spores, which have little or no metabolic activity. Retentostat cultivation has been applied to several bacterial systems including *Escherichia coli* (11), *Paracoccus denitrificans*, and *Bacillus licheniformis* (49) and the autotrophs *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (46, 47). These studies demonstrated that the physiology of these prokaryotes at extremely low specific growth rates could not be accurately predicted by a simple extrapolation of results obtained at higher specific growth rates. In particular, near-zero specific growth rates coincided with increased levels of ppGpp (2), which induces the stringent response, a regulatory program that diverts cellular resources from growth to amino acid biosynthesis (10, 21). Furthermore, it was concluded that extremely slow growth led to a reduction of the maintenance energy requirement of prokaryotes. A recent quantitative analysis on cell retention cultures of *S. cerevisiae* was performed under severely nitrogen-limited growth conditions and used incomplete cell retention (7), which precluded a quantitative comparison with maintenance energy requirements calculated from energy-limited chemostat cultures.

The goal of the present study was to quantitatively analyze the physiology of *S. cerevisiae* at extremely low specific growth rates in glucose-limited retentostat cultures. To this end, an internal filter probe was introduced in the effluent line of standard laboratory chemostat fermentors and used in long-term cultivation runs with complete cell retention. Anaerobic conditions were chosen to facilitate quantification of catabolic fluxes and growth energetics.

#### MATERIALS AND METHODS

**Yeast strain and media.** The prototrophic laboratory strain *S. cerevisiae* CEN.PK113-7D (*MATa MAL2-8<sup>c</sup> SUC2*) was used in the present study. The strain was grown to stationary phase in YPD (yeast-peptone-dextrose) medium (42) at pH 6. After addition of sterile glycerol (20%, vol/vol), 2-ml aliquots were stored in sterile vials at  $-80^{\circ}\text{C}$ . Precultures for chemostat and retentostat cultivation were made by inoculating a frozen stock culture in 500-ml shake flasks with 100 ml of synthetic medium (52) at pH 6 with 2% glucose. To minimize variation in medium composition (and, more especially, in the glucose concentration) during long-term cultivation, 120-liter batches of medium were prepared, filter sterilized, and used for the chemostat and retentostat runs. Vitamins and the anaerobic growth factors ergosterol (final concentration,  $10\text{ mg} \cdot \text{liter}^{-1}$ ) and Tween-80 (final concentration,  $420\text{ mg} \cdot \text{liter}^{-1}$ ) were added to the medium reservoirs prior to inoculation, as described previously (53). At the same time, 1.5 ml of a 20% (wt/wt) solution of the antifoaming agent Struktol J673 (Schill and Seilacher AG, Hamburg, Germany), sterilized separately at  $120^{\circ}\text{C}$ , was added per liter of complete medium.

**Chemostat cultivation.** Triplicate anaerobic chemostat cultivations were performed at a dilution rate of  $0.025\text{ h}^{-1}$  in 2-liter fermentors (Applikon, Schiedam, The Netherlands) with a stirrer speed of 800 rpm. The working volume was kept at 1.4 liters by means of an electrical level sensor. To maintain anaerobic conditions, both the fermentor and the medium vessel were sparged with  $\text{N}_2$  (5.0; Linde Gas Benelux, The Netherlands) at a flow rate of  $0.70\text{ liters} \cdot \text{min}^{-1}$  and ca.  $5\text{ ml} \cdot \text{min}^{-1}$ , respectively. To prevent oxygen diffusion, fermentors were equipped with Norprene tubing (Saint-Gobain Performance Plastics, Charny, France) and Viton O-rings (Eriks, Alkmaar, The Netherlands). Temperature was kept constant at  $30^{\circ}\text{C}$ , and the pH was controlled at 5.0 by automatic addition of 2 M potassium hydroxide. Steady state was defined as the situation in which at

least five volume changes had passed since the last change in culture parameters and in which the biomass dry weight and all production and consumption rates had remained constant ( $<2\%$  variation) for at least two volume changes. Culture purity was routinely checked by phase-contrast microscopy and by plating on synthetic medium agar containing  $20\text{ g} \cdot \text{liter}^{-1}$  glucose and 20 mM lithium chloride (12).

**Retentostat cultivation.** The autoclavable AppliSense sample filter assembly (Applikon, Schiedam, The Netherlands) consists of a hydrophobic tubular polypropylene filter with a pore size of  $0.22\text{ }\mu\text{m}$  and a stainless steel hollow filter support. Before use, the filter was wetted by incubation for 15 h in 96% ethanol. The filter was then thoroughly rinsed with a buffer solution ( $8.0\text{ g} \cdot \text{liter}^{-1}$  NaCl,  $0.2\text{ g} \cdot \text{liter}^{-1}$  KCl,  $1.0\text{ g} \cdot \text{liter}^{-1}$   $\text{Na}_2\text{HPO}_4$ ,  $0.2\text{ g} \cdot \text{liter}^{-1}$   $\text{KH}_2\text{PO}_4$ ) and inserted in the fermentor prior to autoclaving. After reaching steady state, the fermentors were switched from chemostat to retentostat mode by withdrawing the effluent through the AppliSense sample filter assembly instead of using the standard effluent tube. Retentostat cultures were operated at a dilution rate of  $0.025\text{ h}^{-1}$  and sparged with nitrogen at the same rate as the chemostat cultures to maintain anaerobic conditions. As withdrawal of samples could disturb biomass accumulation inside the retentostat, sample volume and sampling frequency were minimized. To this end, fermentors were fitted with in-house manufactured sampling ports with minimal dead volumes of  $<5\text{ ml}$ , and samples were weighed to accurately quantify culture loss. Throughout the retentostat runs, the impact of sampling on calculated specific growth rates was less than 2% (calculations not shown).

**Gas analysis.** The exhaust gas from chemostat and retentostat cultures was cooled with a condenser ( $2^{\circ}\text{C}$ ) and dried with a PermaPure Dryer (model MD 110-48P-4; Inacom instruments, Veenendaal, The Netherlands) prior to online analysis of carbon dioxide with a Rosemount NGA 2000 Analyser (Baar, Switzerland).

**Substrate, metabolites, and biomass determination.** Culture dry weight was measured according to Postma et al. (37). After centrifugation of the culture broth (at room temperature for 3 min at  $16,000 \times g$ ), supernatants were analyzed by high-performance liquid chromatography (Waters, Milford, MA) equipped with Galaxie software (Varian Inc., Palo Alto, CA). The Aminex HPX-87H column (Bio-Rad, Hercules, CA) was eluted with sulfuric acid ( $5\text{ mM}$ ;  $0.6\text{ ml} \cdot \text{min}^{-1}$ ) at  $59^{\circ}\text{C}$ . Detection was by means of a dual-wavelength absorbance detector (Waters 2487) and a refractive index detector (Waters 2410). Succinate, acetate, D-lactate, and residual glucose were also measured enzymatically (Boehringer Roche Diagnostics GmdH, Mannheim, Germany). Samples for analysis of residual glucose were rapidly quenched with cold steel beads (29). For analysis of trehalose and glycogen, exactly 20 ml of culture broth was centrifuged (at  $4^{\circ}\text{C}$  for 5 min at  $10,000 \times g$ ), washed with cold demineralized water, and finally suspended in an exact volume to obtain samples with  $5.00\text{ g} \cdot \text{liter}^{-1}$  biomass and stored at  $-20^{\circ}\text{C}$ . Trehalose and glycogen measurements were performed as described by Parrou and Francois (32). Glucose released by glycogen and trehalose conversion was determined using the UV method based on Roche kit number 0716251 (Almere, The Netherlands). Trehalose and glycogen amounts were determined in triplicate measurements for each sample.

**Assessment of culture viability.** The numbers of CFU were counted by sampling 5 ml of culture broth into 20 ml of 10 mM Na-HEPES buffer, pH 7.2, with 2% glucose. Tenfold dilution series (5 to 7 dilutions) were made in 0.1% (wt/wt) peptone and plated in triplicate on 2% (wt/vol) YPD plates with Bacto agar (Becton Dickinson and Co. Sparks, MD). After 2 days of incubation at  $30^{\circ}\text{C}$ , at least 200 colonies were counted. The total cell concentration was measured with a Coulter counter using a  $50\text{-}\mu\text{m}$  orifice (Multisizer II, Beckman, Fullerton, CA) and used to calculate the fraction of viable cells.

**Viability stains.** A commercial Live/Dead Yeast viability kit (Invitrogen, Carlsbad, CA) was used to estimate the fraction of vital cells in culture samples. Culture samples (5 ml) were diluted with 20 ml of 10 mM Na-HEPES buffer (pH 7.2) with 2% glucose. One milliliter of this suspension was centrifuged at  $16,060 \times g$  for 5 min. The pellet was subsequently dissolved in 1 ml of incubation buffer (10 mM Na-HEPES buffer, pH 7.2, 2% glucose). One microliter of Fun-1 dye (10 mM in dimethyl sulfoxide) was added, and the cell suspension was incubated for 1 h at  $30^{\circ}\text{C}$ . Metabolically active cells were identified and counted based on the formation of red cylindrical intravacuolar structures revealed by fluorescence microscopy (Imager-D1; Carl-Zeiss, Oberkochen, Germany) equipped with Filter Set 09 (fluorescein isothiocyanate long-pass filter; band-pass filter with excitation at 450 to 490 nm; 510-nm beam splitter [dichroic mirror]; long-pass filter with emission at 515 nm; Carl-Zeiss). At least 200 cells were counted. Standard deviation was typically below 7%. To assess cell lysis, the concentration of proteins released in culture supernatants was assayed by a Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (fatty acid free; Sigma) as a standard.

**Prediction of retentostat growth kinetics.** Growth kinetics in retentostat cultures were predicted with two mass balance equations, one for biomass (equation 1, which assumes complete cell retention and constant volume) and one for substrate (equation 2). In these equations,  $C_x$  denotes the biomass concentration ( $\text{g} \cdot \text{liter}^{-1}$ ),  $\mu$  is the specific growth rate ( $\text{h}^{-1}$ ),  $C_s$  is the residual glucose concentration ( $\text{g} \cdot \text{liter}^{-1}$ ),  $C_{s,\text{in}}$  is the glucose concentration in the feed ( $\text{g} \cdot \text{liter}^{-1}$ ),  $D$  is the dilution rate ( $\text{h}^{-1}$ ), and  $q_s$  is the specific glucose consumption rate ( $\text{g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ).

$$\frac{dC_x}{dt} = \mu \cdot C_x \quad (1)$$

$$\frac{dC_s}{dt} = D(C_{s,\text{in}} - C_s) - q_s \cdot C_x \quad (2)$$

The specific substrate consumption rate can be described by the Herbert-Pirt (19, 36) equation (equation 3), including glucose consumption for maintenance at zero growth, in which  $Y_{\text{sx}}^{\text{max}}$  is the maximum yield of biomass on substrate ( $\text{g} \cdot \text{g}^{-1}$ ) and  $m_s$  is the maintenance coefficient ( $\text{g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ).

$$q_s = \frac{\mu}{Y_{\text{sx}}^{\text{max}}} + m_s \quad (3)$$

The retentostat cultivation is performed under glucose limitation and because  $C_s$  is much less than  $C_{s,\text{in}}$ , a pseudo steady state can be assumed, i.e.,  $dC_s/dt = 0$ . When equation 3 is substituted in equation 2, an expression for  $\mu$  can be obtained, which can be substituted in equation 1, which after integration leads to equation 4:

$$C_x(t) = \left( C_{x,0} - \frac{D(C_{s,\text{in}} - C_s)}{m_s} \right) \cdot e^{-m_s \cdot Y_{\text{sx}}^{\text{max}} \cdot t} + \frac{D(C_{s,\text{in}} - C_s)}{m_s} \quad (4)$$

Equation 4 describes an ideal situation in which no loss of viability occurs.

**Calculation of fluxes from concentrations in retentostat cultures.** The volumetric fluxes ( $r$ ) of products ( $i$ ) were calculated with the mass balance equation:

$$r_i = \frac{dC_i}{dt} - D(C_{i,\text{in}} - C_i) \quad (5)$$

The measured metabolite concentrations ( $C_i$   $\text{mmol} \cdot \text{liter}^{-1}$ ) were plotted in Matlab (The MathWorks, Natick, MA) and fitted with a linear or polynomial (third order) equation in order to calculate the derivative,  $dC_i/dt$ , using Matlab Basic fitting. Curve fits were inspected visually by plotting the residuals. The square of the Pearson product moment correlation coefficient ( $R^2$ ) calculated with the RSQ function from Excel (Microsoft, Seattle, WA) was above 0.93 for all fits, except for lactate where the  $R^2$  was 0.45. However,  $dC_i/dt$  is at least 20-fold lower than  $D \cdot C_i$ ; therefore, the influence on the flux is very small. To account for evaporation, the volumetric production of ethanol was calculated from the production of carbon dioxide after subtraction of  $\text{CO}_2$  produced for the formed acetate (1 mol per mole of acetate) and biomass (5.85 mmol per gram of biomass [53]).

The viability of the cultures was measured, and specific fluxes were corrected by assuming that only the viable biomass was metabolically active (equation 6):

$$q_i = \frac{r_i}{C_{x,\text{viable}}} \quad (6)$$

**Calculation of specific growth rate.** To calculate specific growth rate in the retentostat cultures, the measured total biomass concentrations (which include viable as well as nonviable cells) were fitted with  $C_x = A \cdot e^{B \cdot t} + C$ , which is of the same shape as equation 4 (Matlab; function fminsearch for minimizing sum of squares of errors by varying  $A$ ,  $B$ , and  $C$ ). Having determined the coefficients  $A$ ,  $B$ , and  $C$ , the derivative ( $dC_x/dt$ ) could be calculated. Only the viable biomass can replicate, and by assuming that no lysis (i.e., loss of measurable biomass) occurs, the specific growth rate was calculated with equation 7:

$$\mu = \frac{dC_{x,\text{total}}/dt}{C_{x,\text{viable}}} \quad (7)$$

## RESULTS

**Maintenance energy requirement in anaerobic chemostat cultures.** The maintenance energy requirement has a profound impact on growth at low specific growth rates. To estimate the

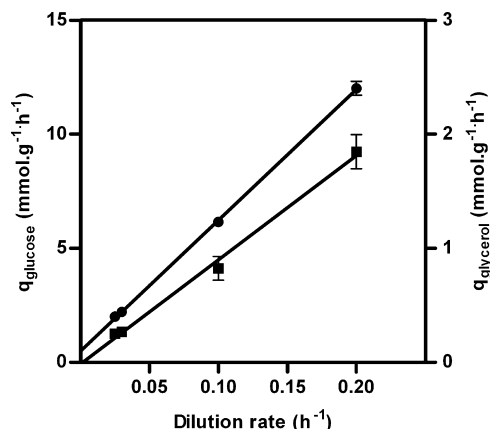


FIG. 1. Specific rates of glucose consumption (●) and glycerol production (■) in anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D. Each data point represents the average of data from three independent chemostat cultures; error bars indicate the standard deviations.

maintenance energy requirement of *S. cerevisiae* CEN.PK113-7D in anaerobic, glucose-limited cultures, a set of reference chemostat cultures was run at different specific growth rates (0.025 to 0.20  $\text{h}^{-1}$ ). In these chemostat cultures, a linear relationship was found between the specific growth rate and the specific rate of glucose consumption (Fig. 1). This relationship is consistent with a growth rate-independent maintenance energy requirement (35). The maintenance energy coefficient  $m_s$  calculated from these experiments was 0.50  $\text{mmol}$  of glucose  $\text{g}^{-1}$  of biomass  $\text{h}^{-1}$ . Since the anaerobic dissimilation of glucose via alcoholic fermentation yields 2 mol of ATP per mole of glucose, this is equivalent to a maintenance requirement for ATP ( $m_{\text{ATP}}$ ) of 1  $\text{mmol}$  of ATP  $\text{g}^{-1}$  of biomass  $\text{h}^{-1}$ . This value is in good agreement with an early publication on microaerobic growth of a respiratory-deficient *S. cerevisiae* strain (40). The maximum biomass yield corrected for maintenance ( $Y_{\text{sx}}^{\text{max}}$ ) calculated from the data shown in Fig. 1 was 0.097 g of biomass  $\text{g}^{-1}$  of glucose, which is in good agreement with previously reported data for another *S. cerevisiae* strain (53).

In anaerobic cultures of *S. cerevisiae*, carbon dioxide is formed not only by alcoholic fermentation but also in biosynthetic reactions. This net oxidation of glucose in biosynthesis is coupled to the formation of NADH, which needs to be reoxidized by glycerol production (4, 48). Indeed, the specific rate of glycerol production in the anaerobic chemostat cultures was linearly correlated with the specific growth rate. Extrapolation of the data to a specific growth rate of 0 suggested that glycerol formation will be absent in nongrowing cultures (Fig. 1).

**Implementation of anaerobic retentostat cultivation.** For retentostat cultivation of *S. cerevisiae*, a filter probe, originally developed for in-line analysis of extracellular metabolite concentrations, was introduced into laboratory fermentors that were also equipped with rapid sampling ports with a minimized dead volume (see Materials and Methods section). In trial experiments, the choice of antifoaming agent was identified as a crucial factor in the use of this setup. Silicone antifoams resulted in premature clogging of the polypropylene filter, but



the antifoam Struktol J163 allowed for long-term operation (>30 days) of the retentostats. It was empirically determined that a flow rate of 35 ml h<sup>-1</sup> could be stably maintained. At a working volume of 1.4 liters, this resulted in a dilution rate of 0.025 h<sup>-1</sup>, which enabled a smooth transition from steady-state chemostat cultivation at this dilution rate. Regular plating of effluent samples confirmed that use of the filter probe resulted in complete cell retention.

#### Growth, viability, and maintenance in retentostat cultures.

If, even at near-zero specific growth rates, the maintenance energy requirement is independent of the specific growth rate, biomass accumulation in a retentostat can be predicted based on estimations of  $m_s$  and  $Y_{SX}^{\max}$  in chemostat cultures. The accumulation of biomass should then follow equation 4 (see Materials and Methods). The actual accumulation of biomass closely corresponded to the prediction during the first 7 days of retentostat cultivation (Fig. 2A). During this period, the specific growth rate decreased from 0.025 h<sup>-1</sup> to <0.004 h<sup>-1</sup> (Fig. 2B). According to equation 4, the increase of the biomass concentration in the retentostat cultures should level off after prolonged cultivation. In practice, however, the biomass concentration continued to increase (Fig. 2A).

A possible explanation for the increase of the biomass concentration during prolonged retentostat cultivation was that part of the biomass was not consuming glucose for growth or maintenance, thereby enabling the remaining biomass in the culture to grow. To investigate this hypothesis, the viability of the cultures was monitored. Viability assays by both a commercial fluorescence microscopy kit and CFU counts indicated a partial loss of viability during long-term retentostat cultivation. The fluorescence microscopy kit indicated a decrease in the viability from 91% ± 8% in the chemostat cultures to 79% ± 6% after 22 days of retentostat cultivation (Fig. 2C). CFU counts confirmed a decrease of culture viability although the loss of viability indicated by this method was more pronounced (ca. 60% viability after 22 days) (Fig. 2C). Since loss of reproductive capacity during plating may have led to an underestimation of culture viability in the CFU assays, the fluorescence microscopy estimations were used in further calculations. Consistent with the occurrence of cell death, extracellular protein concentrations in the retentostat cultures increased over time (Fig. 2C).

When specific growth rates were calculated based on the viable fraction of the biomass in the retentostat cultures (Fig. 2A), they decreased to below 0.001 h<sup>-1</sup> after 22 days of cultivation, corresponding to a doubling time of over 650 h (Fig. 2B). The accumulation of viable biomass agreed closely with the pattern predicted by equation 4, indicating that, also at near-zero specific growth rates, the biomass yield of *S. cerevisiae* can be adequately predicted based on a growth-independent maintenance energy requirement. Indeed, the specific rate of glucose consumption in the retentostat cultures asymptotically approached the value of  $m_s$  estimated from the chemostat experiments (Fig. 3A).

Residual glucose concentrations, analyzed after rapid sampling from the retentostats, decreased during prolonged cultivation. A linear relationship was observed between the specific rate of glucose consumption and the residual glucose concentration ( $C_s$ ). High-affinity glucose transporters in *S. cerevisiae* have a substrate saturation constant ( $K_m$ ) for glucose of ca. 1

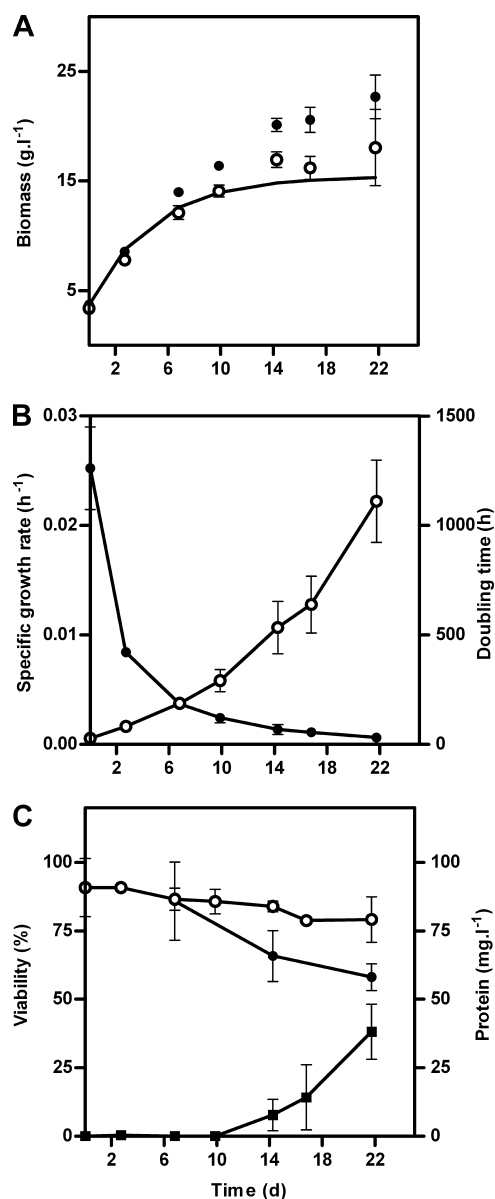


FIG. 2. Growth and viability of *S. cerevisiae* CEN.PK113-7D in retentostat cultures. A steady-state anaerobic chemostat culture ( $D = 0.025 \text{ h}^{-1}$ ) was switched to retentostat mode at time zero. Data points represent average  $\pm$  mean deviation of measurements of two independent cultures. (A) Total biomass concentration (●) and viable biomass concentration (estimated from fluorescent staining) (○). (B) Specific growth rate (●) and doubling time (○) during retentostat cultivation. (C) Culture viability assayed by means of CFU count (●) and fluorescent staining (○); extracellular protein concentration (■) is also shown.

mM (6, 39). At the low substrate concentrations in the retentostat cultures, these transporters operate very far below substrate saturation ( $C_s \ll K_m$ ). When simple Michaelis-Menten kinetics are assumed for extracellular glucose, this resulted in a linear relationship between  $C_s$  and transport rate:  $V_{\text{transport}} = (V_{\text{max,transport}}/K_m) \times C_s$ . The results are therefore consistent with a situation in which kinetics of substrate consumption are primarily determined by high-affinity glucose transport.

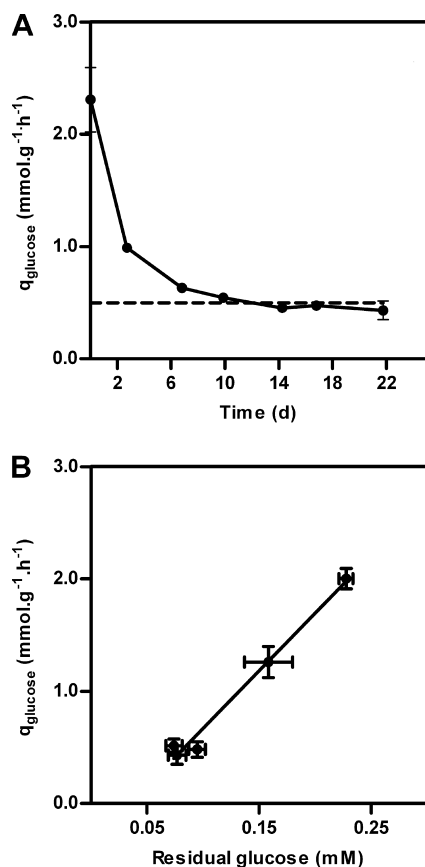


FIG. 3. Kinetics of glucose consumption in retentostat cultures of *S. cerevisiae* CEN.PK113-7D. A steady-state anaerobic chemostat culture ( $D = 0.025 \text{ h}^{-1}$ ) was switched to retentostat mode at time zero. Data points represent average  $\pm$  mean deviation of measurements on two independent cultures. (A) Specific glucose consumption rate ( $q_s$ ) by the viable biomass in the cultures. The dashed line indicates the maintenance coefficient ( $m_s$ ) calculated from chemostat experiments. (B) Relationship between the specific glucose consumption rate ( $q_s$ ) and the residual glucose concentration in the retentostat cultures.

**Metabolic fluxes.** Theoretically, prolonged cultivation in a retentostat should lead to a situation in which all glucose consumed by the cultures is dissimilated to meet maintenance energy requirements. In anaerobic cultures of *S. cerevisiae*, this would imply quantitative conversion of glucose to ethanol and carbon dioxide. Indeed, during long-term retentostat cultivation, the specific rates of alcoholic fermentation decreased to stabilize at a value close to the  $m_{\text{ATP}}$  calculated from the chemostat experiments (Fig. 4A).

The strict linear correlation between glycerol production and specific growth rate that was observed in chemostat cultures ( $\mu$  of  $\geq 0.025 \text{ h}^{-1}$ ) (Fig. 1) was not maintained at the extremely low specific growth rates in the retentostat cultures (Fig. 5). Even when, after 22 days of retentostat cultivation, the specific growth rate had decreased to below  $0.001 \text{ h}^{-1}$ , a specific rate of glycerol production of  $0.04 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$  was observed (Fig. 4B). This is over sevenfold greater than would be predicted based on the constant ratio between glycerol production and specific growth rate observed in the chemostat cultures. In addition to glycerol formation, the retentostat cul-

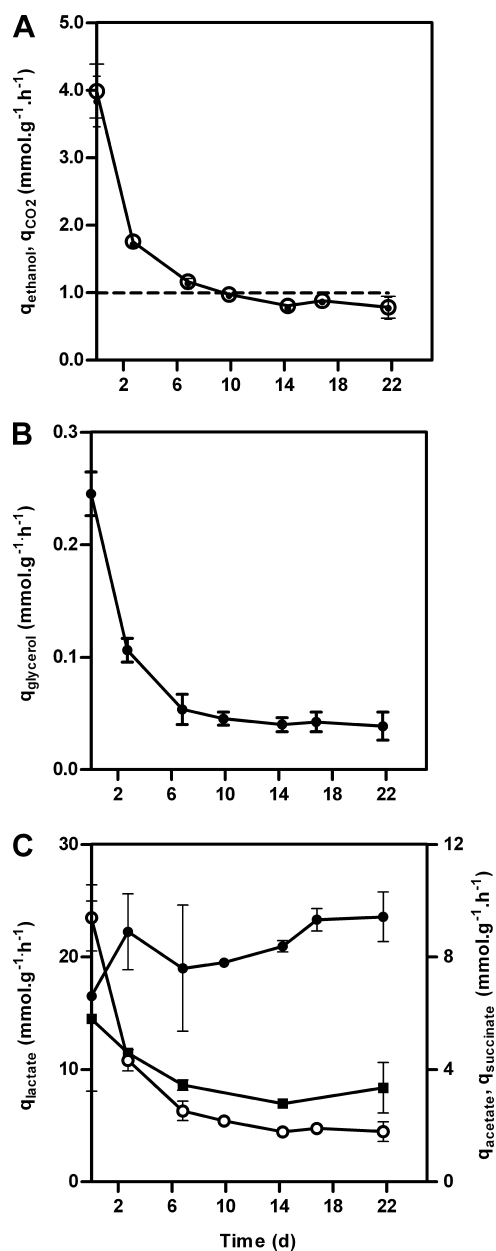


FIG. 4. Product formation in retentostat cultures of *S. cerevisiae* CEN.PK113-7D. A steady-state anaerobic chemostat culture ( $D = 0.025 \text{ h}^{-1}$ ) was switched to retentostat mode at time zero. Data points represent average  $\pm$  mean deviation of measurements on two independent cultures. (A) Specific rates of ethanol (●) and  $\text{CO}_2$  (○) production. The dashed line indicates complete conversion of glucose into these two products. (B) Specific rates of glycerol (●) production. (C) Specific rates of acetate (●), D-lactate (○), and succinate (■) production.

tures also produced D-lactate, acetate, and succinate at low but significant rates (Fig. 4C). The specific rate of D-lactate production was linearly correlated with the specific rate of glucose consumption. This is consistent with the notion that D-lactate production via the methylglyoxal bypass is strongly correlated with glycolytic flux, probably via the intracellular concentration of its precursor dihydroxyacetone phosphate (28). Production

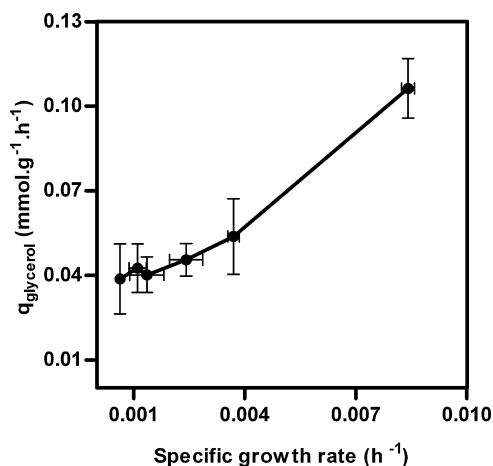


FIG. 5. Specific rates of glycerol production (●) in anaerobic glucose-limited retentostat cultures of *S. cerevisiae* CEN.PK113-7D, plotted as a function of specific growth rate. Data points represent average  $\pm$  mean deviation of measurements on two independent cultures.

of succinate, which is a known minor by-product of anaerobic *S. cerevisiae* cultures, also decreased with a decreasing specific growth rate. Interestingly, specific production rates of acetate were not clearly correlated with the specific growth rate. Formation of acetate and, depending on the biochemical pathway used for its formation, succinate generates NADH that may contribute to the formation of glycerol. Carbon balances over the retentostat cultures that included glucose, biomass,  $\text{CO}_2$ /ethanol, glycerol, acetate, lactate, and succinate yielded an average carbon recovery of  $98\% \pm 5\%$ .

In chemostat cultures of *S. cerevisiae*, accumulation levels of glycogen exhibit a negative correlation with the specific growth rate (17, 32, 43). To investigate whether increased accumulation of storage carbohydrates also occurred at near-zero specific growth rates, glycogen and trehalose contents were measured in biomass samples from the steady-state chemostat cultures ( $D$  of  $0.025 \text{ h}^{-1}$ ) and from 22-day retentostat cultures. Indeed, glycogen contents increased from  $4.3\% \pm 0.8\%$  (glucose equivalents/biomass) in the chemostat cultures to  $9.1\% \pm 0.6\%$  in the retentostat cultures. The glycogen content of the retentostat-grown cells was close to that of cells from nitrogen-starved, glucose-grown shake-flask cultures ( $13\% \pm 1\%$ ). The trehalose contents of the chemostat and retentostat samples did not differ significantly ( $1.0\% \pm 0.4\%$  under both conditions).

## DISCUSSION

**Maintenance requirements and growth kinetics in retentostat cultures.** Biomass accumulation and specific rates of glucose consumption at near-zero growth rates in the retentostat cultures could be accurately predicted based on a maintenance coefficient calculated from chemostat cultures grown at higher specific growth rates ( $0.025$  to  $0.20 \text{ h}^{-1}$ ). This indicates that, in contrast to several prokaryotes that exhibit a stringent response (2, 3, 49), *S. cerevisiae* does exhibit a reduced maintenance energy requirement at very low specific growth rates. Further indications that glucose metabolism in *S.*

*cerevisiae* has not specifically evolved for slow growth under sugar limitation were obtained from the kinetics of glucose consumption in the retentostat cultures. During long-term retentostat cultivation, the residual glucose concentration decreased to a value of ca.  $0.08 \text{ mM}$  (Fig. 3). This concentration represents the most accurate estimate of the threshold concentration for anaerobic growth of *S. cerevisiae* on glucose as the sole carbon source as even lower concentrations will no longer enable the yeast cells to sustain a glycolytic flux that is sufficiently high to meet maintenance energy requirements. Although much lower than the substrate saturation constant of the known high-affinity transporters of *S. cerevisiae* (39), this threshold concentration is extremely high in comparison with the  $K_m$  of glucose uptake systems of many other microorganisms. Consequently, *S. cerevisiae* seems to be poorly equipped to compete with other organisms under severely glucose-limited conditions if the outcome of such a competition is predicted from sugar uptake kinetics. Threshold concentrations for individual substrates can be lower during the simultaneous utilization of substrate mixtures (13). However, in view of the narrow range of sugars that support anaerobic growth of *S. cerevisiae*, mixed substrate utilization is unlikely to improve its competitiveness in natural, sugar-limited environments.

The increased accumulation of glycogen observed at near-zero specific growth rates confirms and extends earlier studies on aerobic chemostat cultures (17, 43) in which a negative correlation between specific growth rate and glycogen content of *S. cerevisiae* was found at higher specific growth rates. Although the net fluxes toward glycogen production were very low in comparison with the total rate of glucose consumption, increased accumulation of storage carbohydrates under these severely energy-limited conditions seems counterintuitive as it competes with maintenance energy metabolism. However, from an evolutionary perspective, accumulation of such energy reserves may contribute to the ability of the yeast to rapidly energize protein synthesis upon a relief of energy limitation (43).

The results of this study are compatible with the notion that *S. cerevisiae* has evolved in natural environments where growth is not limited by sugar availability but, for instance, by availability of nitrogen. *S. cerevisiae* may, however, employ strategies to survive and compete during spells of low sugar availability via mechanisms other than an optimization of maintenance metabolism and sugar uptake kinetics. Instead, growth of competing organisms may be inhibited by the production of ethanol and organic acids during sugar excess periods that precede severe glucose limitation. It should be taken into account that the present study was done with a haploid laboratory strain. In diploid or polyploid strains of *S. cerevisiae*, induction of sporulation under conditions of low nutrient availability (45) provides an alternative survival strategy. Retentostat cultivation may offer an attractive tool to quantitatively analyze the relationship between nutrient availability and onset of sporulation.

**Zero-growth product formation.** In an ideal retentostat scenario, a constant rate of purely dissimilatory metabolism, equal to the culture's maintenance energy requirement, as well as complete viability, is maintained over prolonged periods of time. Such a scenario would be optimal for industrial production of typical catabolic products of sugar metabolism such as

ethanol and other microbial fermentation products (7) as it would completely eliminate formation of biomass and growth-associated by-products. In the present study, ethanol production by *S. cerevisiae* was shown to deviate from this ideal behavior in two ways.

First, although over 90% of the glucose carbon was converted into ethanol and carbon dioxide during long-term retentostat cultivation, formation of glycerol was not completely abolished. This was unexpected as glycerol production by anaerobic yeast cultures that are not osmotically stressed is generally regarded as strictly growth related (4, 48). Formation of glycerol was accompanied by the production of small amounts of acetate and succinate. Identification of the pathways responsible for the formation of these metabolites at near-zero growth rates, for example, via isotopomeric labeling studies (50), is essential to interpret their possible roles in turnover of cellular components and cofactor balancing.

Second, a partial loss of viability was observed during long-term retentostat cultivation. This loss was estimated from the fraction of nonviable cells in the cultures. Microscopic examination gave no indications for disintegration of cells, which might have led to the turnover of dead cells by the viable fraction of the cultures (41). Furthermore, we did not observe cell aggregation or flocculation, as reported in a recent report on nitrogen-limited cell recirculation cultivation of a different *S. cerevisiae* strain (7). Further research should identify to what extent loss of viability under near-zero growth conditions is linked to culture heterogeneity, for example, with respect to cell cycle or replicative age (number of budding events that cells have experienced) (for a review, see reference 44). Furthermore, it will be of interest to investigate whether modified expression of genes that have previously been implicated in longevity of yeast cells (for a review, see reference 22) can reduce loss of viability in retentostat cultures.

In industrial settings, the low maintenance-related rates of alcoholic fermentation at near-zero growth rates can be compensated for by maintaining very high biomass concentrations. Additionally, process engineering and/or metabolic engineering may be applied to increase maintenance energy requirements, for example, by addition of uncoupling agents (1, 52) or expression of futile cycles in metabolism (30, 31, 41).

**Use of retentostat cultivation in yeast research.** The setup used for retentostat cultivation described in this publication is easy to implement in laboratory chemostats. Long-term cultivation (up to 60 days [data not shown]) with complete cell retention was possible without clogging of the filter probe. For studies that require large culture samples, it will be necessary to use larger fermentors to prevent disturbance of the growth rate by sampling.

During long-term retentostat cultivation, the cell cycle of the yeast cells has to come to a virtual standstill, and, consequently, the chronological age of the cells will increase. The current techniques for studying quiescence and chronological aging of *S. cerevisiae* cells, which rely on studying deteriorating conditions such as stationary phase or starvation, may complicate interpretation of results (15, 25). The gradual transition from exponential growth to (near) zero growth, the constant supply of glucose for maintenance purposes, and the accurate control of cultivation conditions in retentostats may offer an interesting experimental platform for studies on chronological ageing.

To explore whether retentostats can, indeed, be used to more clearly distinguish between ageing and starvation responses, it will be essential to record genome-wide responses during retentostat cultivation and to compare these responses to those obtained from nutrient-depletion studies.

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## ERRATUM

### Quantitative Physiology of *Saccharomyces cerevisiae* at Near-Zero Specific Growth Rates

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Volume 75, no. 17, p. 5607–5614, 2009. Page 5611, column 2, Figure 4C: The y-axis labels “ $q_{\text{lactate}}$  ( $\text{mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ )” and “ $q_{\text{acetate}}, q_{\text{succinate}}$  ( $\text{mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ )” should read “ $q_{\text{lactate}}$  ( $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ )” and “ $q_{\text{acetate}}, q_{\text{succinate}}$  ( $\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ ),” respectively.