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1) Baker, Monya. "1,500 scientists lift the lid on reproducibility." Nature, no. 533 (May 26, 2016): 452-54. doi:10.1038/533452a.

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ARTICLE

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Uncoupling growth and succinic acid production in an industrial *Saccharomyces cerevisiae* strain

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Abstract

This study explores the relation between biomass-specific succinic acid (SA) production rate and specific growth rate of an engineered industrial strain of *Saccharomyces cerevisiae*, with the aim to investigate the extent to which growth and product formation can be uncoupled. Ammonium-limited aerobic chemostat and retentostat cultures were grown at different specific growth rates under industrially relevant conditions, that is, at a culture pH of 3 and with sparging of a 1:1 CO_2–air mixture. Biomass-specific SA production rates decreased asymptotically with decreasing growth rate. At near-zero growth rates, the engineered strain maintained a stable biomass-specific SA production rate for over 500 h, with a SA yield on glucose of 0.61 mol mol^{-1}. These results demonstrate that uncoupling of growth and SA production could indeed be achieved. A linear relation between the biomass-specific SA production rate and glucose consumption rate indicated the coupling of SA production rate and the flux through primary metabolism. The low culture pH resulted in an increased death rate, which was lowest at near-zero growth rates. Nevertheless, a significant amount of non-viable biomass accumulated in the retentostat cultures, thus underlining the importance of improving low-pH tolerance in further strain development for industrial SA production with *S. cerevisiae*.

KEYWORDS
high CO_2, low pH, near-zero growth, retentostat, succinic acid

1 | INTRODUCTION

Succinic acid (SA), a C4 dicarboxylic acid and intermediate of the tricarboxylic acid (TCA) cycle (Tretter et al., 2016), is used as precursor for manufacturing a wide range of products in the pharmaceutical, agricultural, and food industries, including detergents, fungicides, herbicides, biodegradable polymers, flavors, and food additives (Zeikus et al., 1999). Based on predicted environmental benefits, 2004 and 2010 reports of the US Department of Energy (DOE) mentioned SA as one of the five most promising bio-based chemicals (Bozell & Petersen, 2010; Werpy & Petersen, 2004). The SA market size is expected to reach 710,000 tons by 2020, with a predicted turn-over of USD 1.1–1.3 billion in 2022 (Dessie et al., 2018; Luthfi et al., 2017). To supply this market, bio-based production of SA will become increasingly important as it can provide a sustainable alternative to petrochemical production (Pinazo et al., 2015).

In recent years, the production of SA in industrial fermentation processes has been realized with naturally SA-producing microorganisms as well as with engineered strains (Beauprez et al., 2010; Chen & Nielsen, 2013). BASF/Corbion-Purac achieved a yield of 0.75 mol of SA per mol of glucose from the natural producer *Basfia succiniproducens*, which was isolated from the bovine rumen (Kuhnert et al., 2010). Researchers from KAIST applied metabolic engineering to improve succinic acid production of the capnophilic rumen bacterium *Mannheimia succiniciproducens* (Lee et al., 2002). They obtained succinic acid yields up to 1.64 mol/mol glucose equivalent (Ahn et al., 2016; Lee et al., 2016) and titers up to 134 g/L (Ahn et al., 2020) by accomplishing almost homo-succinic acid production. Myriant applied an engineered *Escherichia coli* strain for large-scale SA production (Ahn et al., 2016). However, SA-producing bacteria may be affected by bacteriophage infections and generally require a neutral culture pH. The latter poses a requirement for alkali titration during fermentation and a subsequent acidification during downstream processing, resulting in massive waste production in the form of gypsum (Abbott et al., 2009). Fermentation at a low pH provides a way to reduce waste production and, thereby, improve process economics and sustainability.

Due to their low pH tolerance and insensitivity to phage infections, engineered yeasts have been intensively studied as microbial SA production platforms. For instance, Bioamber/Mitsui has replaced its SA producing *E. coli* strain with the yeast *Candida krusei* (Jansen & van Gulik, 2014). Since 2012, DSM/Roquette has applied an engineered *Saccharomyces cerevisiae* strain for industrial-scale SA production (Jansen et al., 2013). To reach a high SA yield, the latter strain was genetically modified by overexpression of the reductive branch of the TCA cycle in the cytosol, with further genetic modification focused on the glyoxylate cycle and SA export across the plasma membrane. Because SA production via the reductive part of the TCA cycle involves a net consumption of CO_2, increasing dissolved CO_2 concentrations should increase the driving force for SA biosynthesis via this pathway. It has indeed been reported that enrichment of the aeration gas with CO_2 significantly increased the rate of SA production in engineered strains of *S. cerevisiae* (Jamalzadeh, 2013; Shah, 2016; Zelle et al., 2010). Although the Gibbs free energy change of the synthesis of SA from glucose is negative, the costs of active SA export (2–3 mol ATP per mol SA exported; Taymaz-Nikerel et al., 2013) requires a net input of ATP. Oxygen therefore needs to be supplied during yeast-based SA production to enable ATP production via respiration (Shah, 2016).

In a study on product recycling across yeast membranes at high SA titers, which was based on a scaled-down industrial SA fed-batch fermentation process at low pH (Wahl et al., 2017), significant ^{13}C labeling of the TCA cycle intermediates fumarate, isocitrate, and α-ketoglutarate was observed within 100 s after extracellular addition of ^{13}C labeled SA. This observation indicated that SA rapidly exchanges over the plasma membrane. In this scaled-down fed-batch process, SA production rates declined with decreasing specific growth rates. This observation was attributed to increased product degradation as well as to increased nongrowth associated energy requirements at high SA titers, which left less substrate available for energy-dependent product formation.

From an industrial point of view, fermentative production of SA in the absence of cell growth would be ideal, as it would minimize formation of biomass as a byproduct and maximize the yield of product on a substrate. Such an uncoupling of growth and product formation requires a producing strain which can maintain a high productivity at near-zero specific growth rates. Due to their dynamic nature, fed-batch cultures are not the best option to study relations between specific growth rate and strain performance (Hewitt & Nienow, 2007). In contrast, chemostat cultivation allows studies on microbial physiology at a constant specific growth rate, under well-defined, stable process conditions. However, chemostat cultivation in laboratory bioreactors is impractical at specific growth rates below 0.025 h^{-1}, due to the long time periods needed to reach a steady-state. As an alternative to chemostat cultivation, retentostat cultures have proven to be excellent tools to study the physiology of *S. cerevisiae* and other microorganisms at low to near-zero growth rates under various nutrient limitations (Boender et al., 2009; Ercan et al., 2015; Hakkaart et al., 2020; Liu et al., 2019; Vos et al., 2016).

The goal of the present study is to investigate whether uncoupling of growth and product formation can be accomplished in a heavily engineered, SA high-producing industrial *S. cerevisiae* strain. The strain was grown under industrially relevant conditions, that is, at an elevated CO_2 level to increase the driving force towards SA biosynthesis via the reductive pathway and a culture pH of 3.0 to facilitate downstream processing (Hakkaart et al., 2020). Ammonium-limited cultures were used, in which glucose was present in excess, to avoid competition for glucose between growth and product formation. Ammonium-limited chemostat and retentostat cultures were used to study the physiology of the industrial strain over a range of low to near-zero specific growth rates. This approach enabled a quantitative assessment of the degree to which biomass-specific production of SA and specific growth rate are coupled and to identify goals for further strain engineering to improve uncoupling of growth and product formation in industrial SA production.

2 | MATERIALS AND METHODS

2.1 | Yeast strain and growth media

The engineered SA-overproducing industrial *S. cerevisiae* strain (SUC632) was kindly provided by Royal DSM B.V. Properties and performance of this strain have been described previously (Jansen et al., 2013; Wahl et al., 2017). Working stocks were stored at −80°C in 1 ml aliquots in YPD medium (10 g/L Bacto yeast extract, 20 g/L Bacto peptone, and 20 g/L glucose) containing 30% (v/v) glycerol. The pre-culture medium contained, per liter of demineralized water: 20 g galactose, 2.3 g urea, 3.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 1 ml trace-element solution, and 1 ml vitamin solution (Verduyn et al., 1992). After filter sterilization (Millex® Syringe Filters, 0.22 µm, Merck Millipore), 1 ml of sterilized chalk solution (0.1 g/g $CaCO_3$)

was added per 100 ml pre-culture medium. The medium for batch cultivation contained, per liter of demineralized water: 30 g galactose, 1.0 g $(NH_4)_2SO_4$, 5.3 g K_2SO_4, 3.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 1 ml trace element solution, 1 ml vitamin solution, 1 ml of a solution containing 3 g/L $FeSO_4{\cdot}7H_2O$ and 15 g/L EDTA, 1 ml of biotin solution (1 g/L), and 0.2 g Pluronic 6100 PE antifoaming agent (BASF). The composition of the chemostat feed medium was the same as the medium used for the batch phase, except for the carbon source, which was 30 g/L glucose instead of 30 g/L galactose. For retentostat cultivation concentrations of glucose and $(NH_4)_2SO_4$ were decreased to 18 g/L and 0.1 g/L, respectively.

2.2 | Pre-cultures and aerobic bioreactor cultures

Pre-cultures were initiated by inoculating 500 ml Erlenmeyer flasks containing 200 ml of pre-culture medium with 2 ml of stock culture and incubated for 24 h in an orbital shaker at 30°C and at a rotation speed of 200 rpm (B Braun Certomat BS-1, Sartorius).

Batch, chemostat and retentostat cultivations were carried out in 7 L bioreactors (Applikon) as described previously (Liu et al., 2019).

During batch cultivation, bioreactors were sparged with compressed air (0.04% CO_2). During chemostat and retentostat cultivation, the reactors were sparged with a 1:1 mixture of air and CO_2 (>99.7% purity, Linde Gas Benelux) by using two mass-flow controllers. The pH was maintained at 3.00 ± 0.05 by automatic titration with 2 M KOH or 2 M H_2SO_4. The dissolved-oxygen tension was measured using an autoclavable Clark sensor (Mettler-Toledo GmbH) but was not controlled. Exhaust gas from bioreactors was dried by first passing through a condenser at 4°C and then through a Perma Pure Dryer (Inacom Instruments). CO_2 and O_2 concentrations in the dried gas were measured with a Rosemount NGA 2000 gas analyzer.

Independent duplicate chemostat cultures were carried out at dilution rates of 0.07, 0.058, 0.048, 0.035, and 0.025 h^{-1}. Steady-state was assumed to be achieved when off-gas CO_2 and O_2 concentrations, biomass dry weight, and cell counts changed by less than 3% difference over two consecutive volume changes. After these criteria had been met, samples were withdrawn after 5, 6, and 7 subsequent volume changes for measurement of biomass dry weight, cell number, concentrations of residual substrates, and (by)products and culture viability.

Retentostat cultures were started with a batch cultivation phase, followed by chemostat cultivation at a dilution rate of 0.025 h^{-1}. After reaching steady-state, chemostat cultures were switched to retentostat mode by switching off the chemostat broth-removal system and activating cell-free culture removal through four filtration probes (Applikon) mounted inside the reactor (Liu et al., 2019). The cell-free effluent was pumped in a sterile effluent vessel using a peristaltic pump (Masterflex) controlled by the vessel weight, such that the broth weight was kept at 5.00 ± 0.05 kg. During retentostat cultivation, the dilution rate was maintained at 0.025 h^{-1}. A gradual transition from chemostat to retentostat medium was accomplished by using two feed pumps (Liu et al., 2019). Duplicate retentostats were carried out and samples were withdrawn every 48 h until the cultures were terminated.

2.3 | Determination of biomass dry weight, cell counts, and culture viability

Biomass dry weight was quantified gravimetrically. Total cell counts were measured by a Z2 Coulter counter (50 μm aperture, Beckman). Cell viability of culture samples was determined with a FungaLight™ Yeast CFDA, AM/Propidium Iodide Vitality Kit, and flow cytometry. Detailed descriptions of the dry weight, cell counts, and viability measurements were described previously (Liu et al., 2019).

2.4 | Rapid sampling for metabolite quantification

Cell-free effluent samples for quantification of extracellular metabolites were obtained from a sample port connected to the retentostat filter assembly. Broth samples for intracellular metabolite measurements were withdrawn by a rapid sampling device connected to the bioreactor as described previously (Liu et al., 2019).

2.5 | Quantification of substrates, products, and intracellular metabolites

Ammonium concentrations were analyzed with a Gally Discrete Analyzer (Thermo Fisher Scientific) with a detection limit of 0.02 mg NH_4^+/L. Concentrations of glucose, succinic acid, and by-products (malate, ethanol, glycerol, acetate, and lactate) were quantified by HPLC, using a Bio-Rad HPX-87H 300 column (7.8 mm) as described previously (Liu et al., 2019). Intracellular metabolite concentrations were quantified using isotope dilution mass spectrometry (LC-IDMS/MS and GC-IDMS) with U-^{13}C-labeled yeast cell extract as internal standard (Wu et al., 2005). Detailed descriptions of the mass spectrometry based quantification protocols were published previously (Cipollina et al., 2009; Seifar et al., 2009; van Dam et al., 2002). The adenylate energy charge (AEC) was calculated as described (Liu et al., 2019).

2.6 | Biomass-specific rate calculations

Calculation of specific growth rates (μ, h^{-1}) and specific death rates (k_d, h^{-1}) were performed as described previously (Liu et al., 2019). Biomass-specific glucose and ammonium consumption rates and biomass-specific production rates of succinic acid, malate, ethanol, glycerol, acetate, and lactate were calculated from the primary measurements of substrate and product concentrations and flow rates in gas and liquid phases using the corresponding material balances. Data reconciliation was performed as described by Verheijen (2009).

3 | RESULTS

3.1 | Growth and viability of *Saccharomyces cerevisiae* SUC632 in ammonium-limited chemostat and retentostat cultures at low pH and high CO_2 levels

To investigate whether the specific SA production rate of the engineered *S. cerevisiae* strain SUC632 was related to its specific growth rate, the strain was cultivated under industrially relevant conditions (50% CO_2, pH = 3.0) in ammonium-limited cultures. Specific growth rates between 0.034 and 0.085 h^{-1} were studied in chemostat cultures, while retentostat cultures were applied to obtained quantitative data on strain performance at specific growth rates from 0.034 h^{-1} to near zero. All these continuous cultures were grown under ammonium limitation. In all retentostat and steady state chemostat cultures, concentrations of residual ammonium were below the detection limit, and residual glucose concentrations were higher than the saturation constant of the high-affinity glucose transporters in *S. cerevisiae* (k_m, ca., 1 mM; Boles & Hollenberg, 1997; Reifenberger et al., 1997), indicating that ammonium was indeed the growth-limiting nutrient (Table S1).

In all chemostat cultures, the viability was between 80% and 90% (Figure 1a). As only the viable cell fraction contributes to substrate consumption, growth, and (by)product formation, all specific conversion rates were expressed per gram of viable cells. The steady-state total and viable biomass concentrations slightly increased with decreasing specific growth rate to become stable below a specific growth rate of 0.042 h^{-1} (Figure 1c). A similar profile was observed for total cell counts, while the average dry mass per cell decreased with decreasing specific growth rate (Figure 1e).

As, during retentostat cultivation, the specific growth rate progressively decreased from 0.025 h^{-1} to near zero, culture viability declined from 80% to a stable value of around 25% between 300 and 450 h of cultivation, with a slight further decrease thereafter (Figure 1b). Total biomass dry weight and cell counts increased during retentostat cultivation according to similar patterns Figure 1d,f). The viable biomass concentration leveled off at 2 g/L after 300 h (Figure 1d). The dry mass per cell was stable during retentostat cultivation (Figure 1f) and identical to the value observed in chemostat cultures at a specific growth rate of 0.034 h^{-1}.

The highest specific death rate (k_d = 0.015 h^{-1}) was observed at the highest specific growth rate of 0.085 h^{-1} applied during chemostat cultivation. In the other, slower growing chemostat cultures, k_d was approximately 0.01 h^{-1} (Figure 1g). From the start of the retentostat cultivations, k_d decreased from 0.01 h^{-1} to approximately 0.005 h^{-1}, while the corresponding specific growth rate steeply decreased to stabilize at values below 0.005 h^{-1} (Figure 1h).

3.2 | Uncoupling of growth and succinic acid production

In nitrogen-limited retentostat cultures, the biomass-specific glucose consumption (q_{glc}) and SA production (q_{suc}) rates decreased asymptotically with decreasing specific growth rate (Figure 2a,b). The q_{suc} stabilized at a value of around 3 mCmol/(g viable biomass)/h for specific growth rates between 0.008 and 0.014 h^{-1} (Figure 2b). This result showed that the engineered strain was still capable of producing SA at near-zero specific growth rates. Apart from SA, malate, ethanol, glycerol, and small amounts of acetate and lactate were formed as byproducts. The biomass-specific production rates of these by-products were combined in a "q_{byp}" term and expressed as mCmol per gram of viable biomass per hour. The specific rate of byproduct formation decreased asymptotically with decreasing specific growth rate to relatively low values (1.2 mCmol/(g viable biomass)/h, Figure 2c). Individual production rates for each by-product are provided in Table S2. The combined chemostat and retentostat data showed that biomass-specific SA production rates increased linearly with glucose consumption rate (Figure 2d). This observation strongly suggests a coupling between the SA production rate and the flux through primary metabolism, possibly as a consequence of altered intracellular metabolite concentrations or altered expression levels of key enzymes. Further research is required to elucidate the underlying mechanisms and identify metabolic engineering targets for boosting SA production rates in the absence of growth. During retentostat cultivation, the yield of SA on glucose asymptotically decreased from almost 0.6 Cmol/Cmol to a stable value of around 0.4 Cmol/Cmol after 400 h of cultivation, whereby the specific growth rate had decreased to a value of approx. 0.003 h^{-1} (Figure 3). The fractions of the consumed glucose used for biomass, SA, and byproduct formation were calculated as a function of the specific growth rate, during steady-state chemostat cultivation at specific growth rates of 0.085, 0.067, 0.055, 0.042, 0.034 h^{-1} and pseudo-steady-state retentostat cultivation (μ = 0.003 h^{-1}) after 400 h (Figure 4). Specifically, the fraction of the consumed glucose converted to SA was highest for the condition where growth was virtually absent (μ = 0.003 h^{-1}).

3.3 | Cellular energy status during near-zero growth cultivation

To investigate the energy status of the SA-producing strain at near-zero growth rates, under the severe nitrogen limitation in low-pH retentostat cultures, intracellular levels of adenine nucleotides (ATP, ADP, and AMP), and the AEC and ATP/ADP ratios were analyzed (Figure 5). During retentostat cultivation, intracellular levels of ATP and ADP slowly decreased (Figure 5b,c) and stabilized after about 350 h. Retentostat cultures showed a stable intracellular AMP level that was approximately twofold higher than observed in the preceding chemostat cultures (Figure 5a). Despite these differences in the concentrations of individual adenine nucleotides, the energy charge did not change upon the switch from chemostat to retentostat cultivation and remained at a stable value of approximately 0.8 throughout the retentostat experiments (Figure 5d). The ATP/ADP ratio slowly increased from a value of 1.5–2.0 at the end of the retentostat cultivation (Figure 5e).

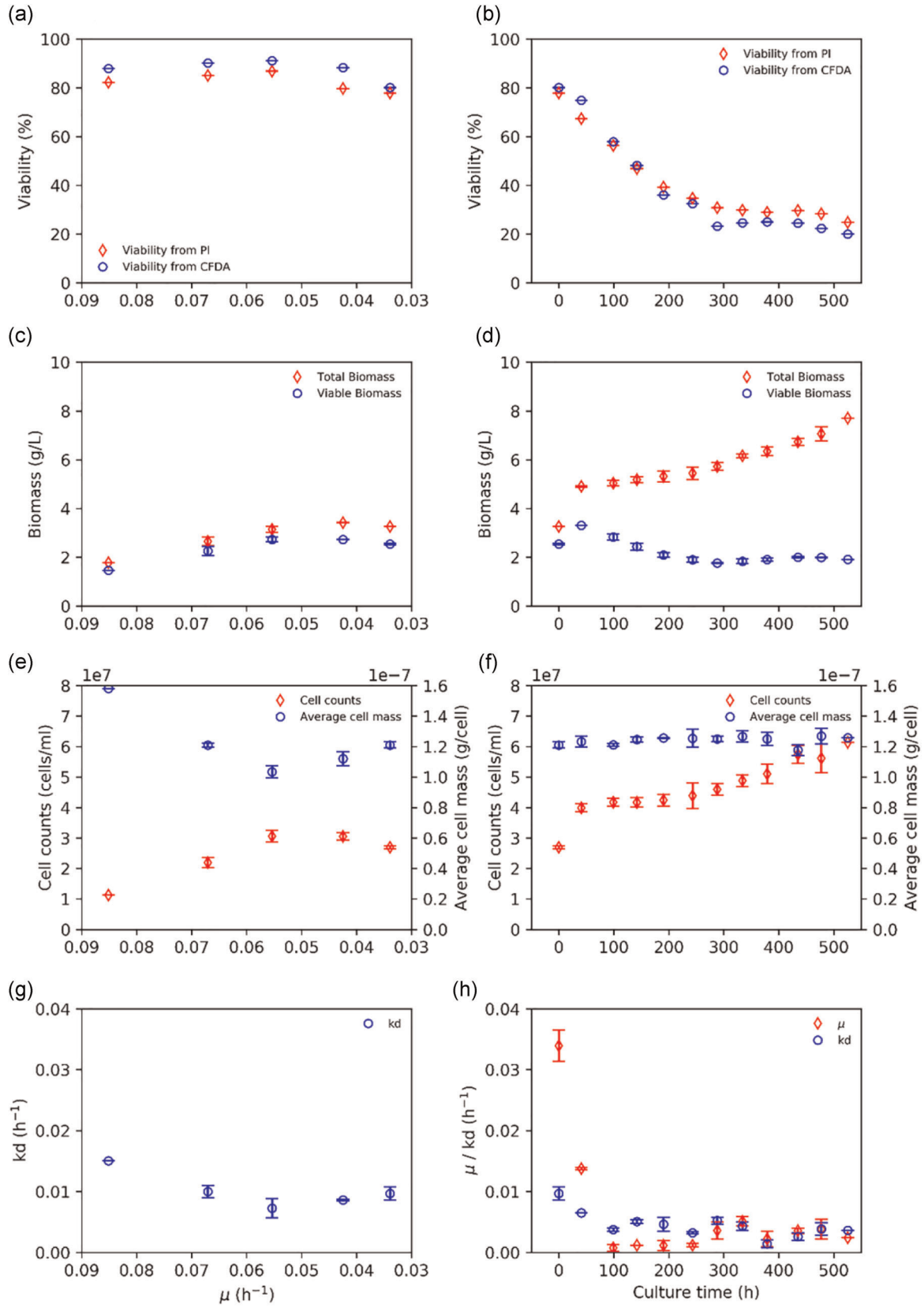

FIGURE 1 Culture viability (a,b), total and viable biomass concentrations (c,d), total cell counts and average cell mass (e,f), and specific growth and death rates (g,h) in aerobic, ammonium-limited chemostat, and retentostat cultures of *Saccharomyces cerevisiae* SUC632. Cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. Left and right panels represent data from steady-state chemostat cultures at different specific growth rates and from retentostat cultures, respectively. In the retentostat cultures, specific growth rates decreased over time (panel H). All data represent averages ± standard errors of data from independent duplicate cultures [Color figure can be viewed at wileyonlinelibrary.com]

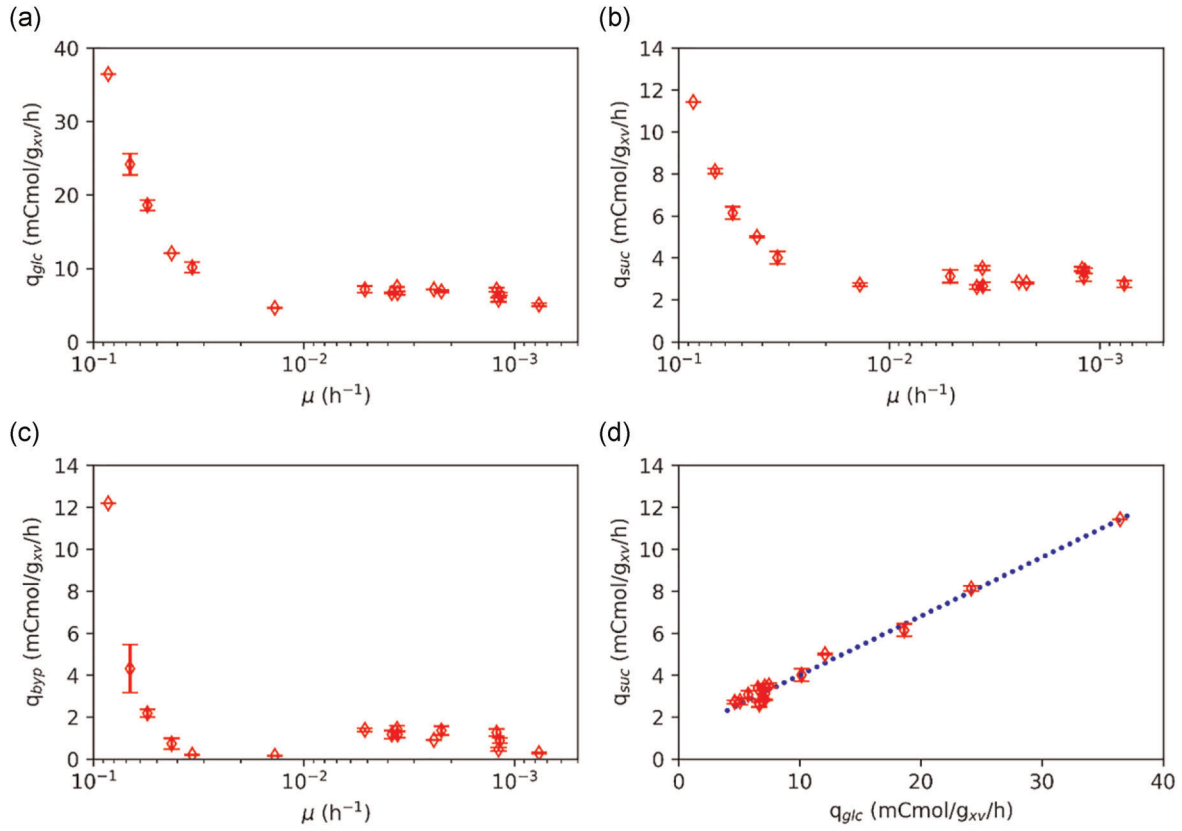

FIGURE 2 Biomass specific consumption rates of glucose (a) and production rates of succinic acid (b) and by-products (c) as a function of the specific growth rate (μ), and specific succinic acid production rate plotted as a function of specific glucose consumption rate (d) in aerobic, ammonium-limited chemostat, and retentostat cultures of *Saccharomyces cerevisiae* SUC632. Cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. q_{byp} represents the sum of the specific production rates of malate, ethanol, glycerol, and acetate. q_{glc} and q_{suc} represent the specific glucose consumption rate and succinic acid production rate, respectively. All data represent averages of results from duplicate chemostat and retentostat cultures with their standard errors [Color figure can be viewed at wileyonlinelibrary.com]

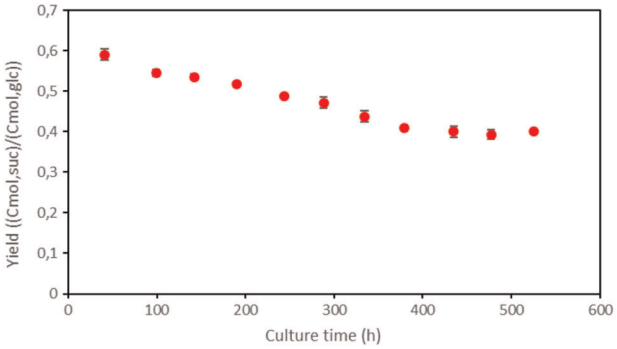

FIGURE 3 Succinic acid yield on glucose during ammonium-limited aerobic retentostat cultivation. Retentostat cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. Specific growth rates at the time points indicated are shown in Figure 1h. All data represent averages with standard errors obtained from duplicate retentostat cultures [Color figure can be viewed at wileyonlinelibrary.com]

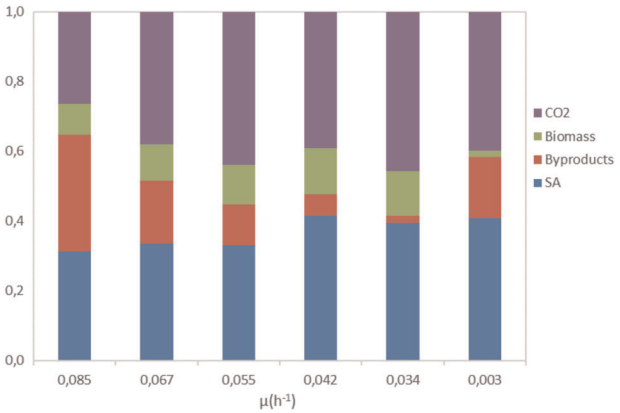

FIGURE 4 Growth rate-dependent division, on carbon basis, of consumed substrate between growth, succinic acid, and byproducts formation in aerobic, ammonium-limited chemostat, and retentostat cultures of *Saccharomyces cerevisiae* SUC632, at different specific growth rates. Cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. The CO_2 fraction was calculated from a total carbon balance [Color figure can be viewed at wileyonlinelibrary.com]

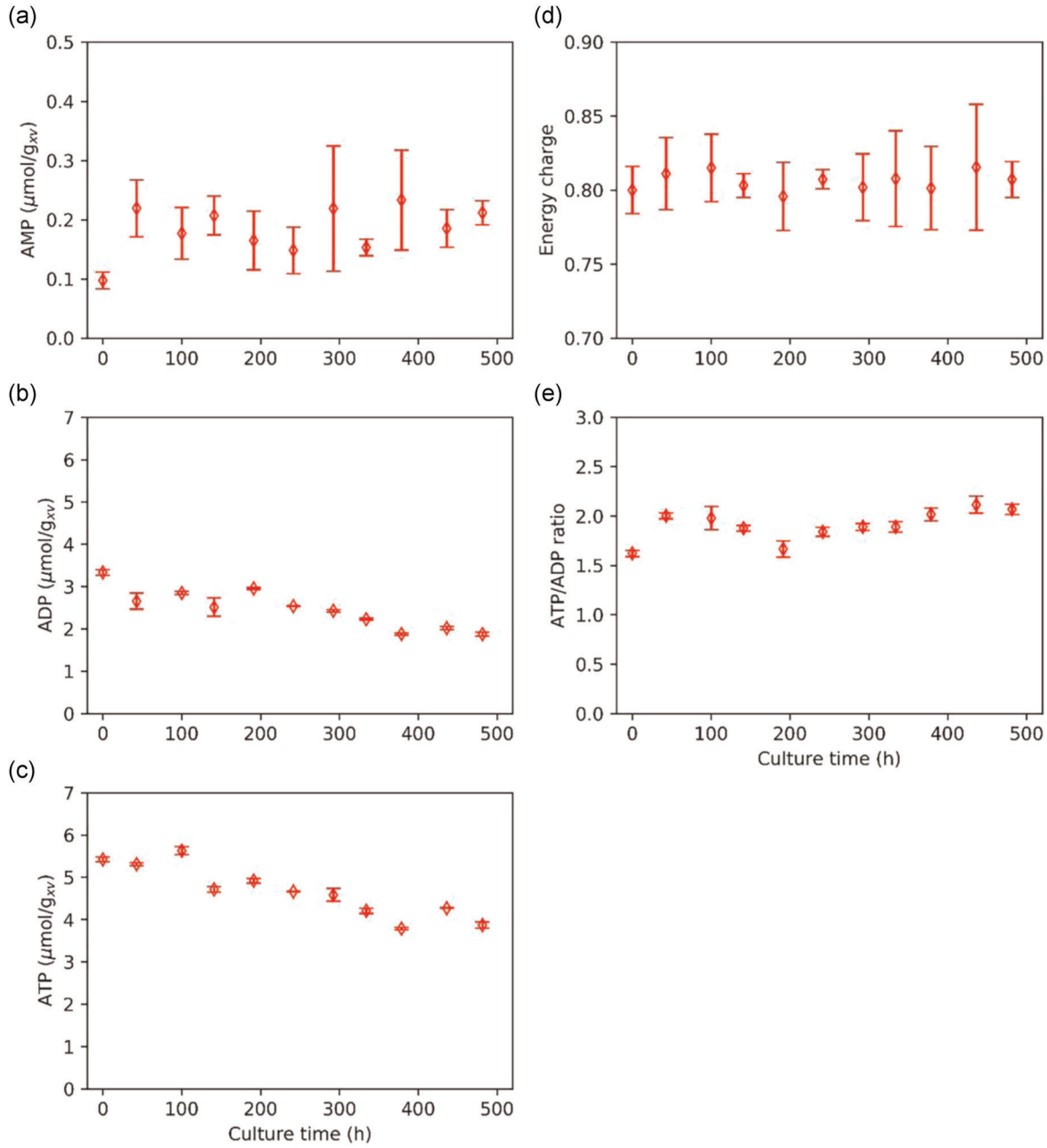

FIGURE 5 Intracellular adenosine phosphate level, adenylate energy charge, and ATP/ADP ratio during aerobic, ammonium-limited retentostat cultivation of *Saccharomyces cerevisiae* SUC632. Retentostat cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. The data at time point $t = 0$ represent the results from the preceding steady-state chemostat cultures at $\mu = 0.034\ h^{-1}$. Specific growth rates at the time points indicated are shown in Figure 1h. Data represent the averages and standard errors of measurements from duplicate cultures [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Levels of the TCA cycle metabolites during retentostat cultivation

The target product in this study, SA, as well as the most important by-product, malate, are both intermediates of the TCA cycle. To assess how SA production correlated with intracellular levels of other TCA-cycle intermediates, intracellular metabolite analyses were performed on the retentostat cultures (Figure 6a–f). The levels of all quantified TCA-cycle intermediates increased during retentostat cultivation of the engineered strain, but followed distinct dynamic patterns. Intracellular levels of citrate, isocitrate, and succinate increased linearly with time, levels of α-ketoglutarate and fumarate decreased until 150 h and increased thereafter, while the malate level increased until 300 h and then decreased. Intracellular levels of citrate, succinate, and malate were two orders of magnitude higher than those of other TCA-cycle intermediates. When compared to a wild-type strain grown at similar glucose consumption rates in aerobic, glucose-limited chemostat cultures (Canelas et al., 2011) levels of citrate, succinate, and

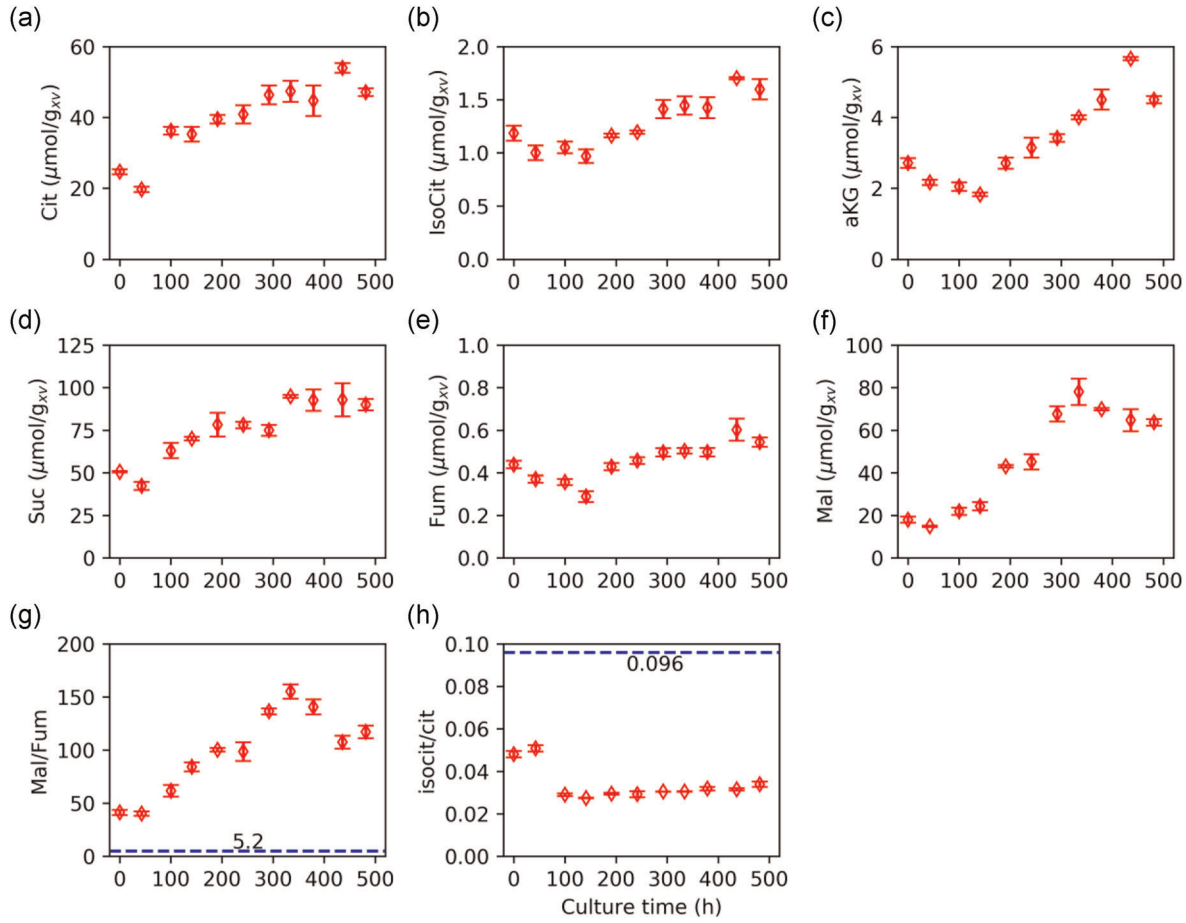

FIGURE 6 Intracellular levels of the TCA cycle metabolites (a–f) and mass action ratio's of fumarase and aconitase (g,h) during aerobic, ammonium-limited retentostat cultivation of SUC632. Retentostat cultures were grown on glucose, at pH of 3 and were sparged with a 1:1 mixture of air and CO_2. Specific growth rates at the time points indicated are shown in Figure 1h. The data at time point $t = 0$ represent the results from the preceding steady-state chemostat cultures at $\mu = 0.034\ h^{-1}$. Dashed blue lines in Figures g and h represent the apparent in vivo equilibrium constants determined for *Saccharomyces cerevisiae* (Canelas et al., 2011). Data represent the averages and standard errors of measurements from duplicate cultures. αKG, α-ketoglutarate; Cit, citrate; Fum, fumarate; IsoCit, isocitrate; Mal, Malate; Suc, succinic acid; TCA, tricarboxylic acid [Color figure can be viewed at wileyonlinelibrary.com]

malate were 5-, 90-, and 25-fold higher, respectively, toward the end of the nitrogen-limited retentostat cultures. Remarkably, the mass action ratio of the fumarase reaction (Figure 6g) was far above the apparent in vivo equilibrium constant of 5.2 (Canelas et al., 2011). This observation suggests that the overall flux was from malate to fumarate, with a low fumarate level resulting from the action of the heterologous, cytosolically expressed NADH-dependent fumarate reductase (FRDg) from *Trypanosoma brucei* (Jansen et al., 2013). Succinate formation via this reaction is highly exergonic, thus providing a plausible explanation for the high intracellular succinate and low fumarate levels. The expression of isocitrate lyase (KIICL1) from *Kluyveromyces lactis* (Jansen et al., 2013) may be responsible for the high intracellular citrate levels as this enzyme, together with aconitase, allows for the conversion of succinate via isocitrate to citrate and vice versa. The observation that the mass action ratio for the aconitase reaction was well below the estimated in vivo K_{eq}, (Figure 6h), indicate that net flux through this enzyme was in the direction of isocitrate.

4 | DISCUSSION

Fermentation processes at near-zero growth rate theoretically allow for highly efficient conversion of substrate to product, as no carbon and energy source is required for biomass formation. However, long-term cultivation of *S. cerevisiae* at the near-zero growth rate in retentostat cultures has been reported to result in accumulation of nonviable, nonproducing cells (Boender et al., 2009; Vos et al., 2016). A previous study with the nonproducing laboratory reference strain CEN.PK113-7D (Hakkaart et al., 2020) showed that, at pH of 3, the specific death rates (k_d) in glucose- and ammonium-limited retentostat cultures (0.0039 ± 0.0005 and $0.0030 \pm 0.0004\ h^{-1}$, respectively) were approximately eightfold higher than in corresponding cultures grown at pH of 5. However, cultivation of the reference strain at near-zero growth rate as such did not result in an increased k_d, because the death rates in glucose- and ammonium-limited retentostat cultures performed at pH of 3 were respectively two and tenfold lower than in corresponding

chemostat cultures grown at a specific growth rate of 0.025 h^{-1} at pH of 3 (Hakkaart et al., 2020).

In the present study, ammonium-limited steady-state chemostat cultivation at a specific growth rate of 0.025 h^{-1}, under industrially relevant conditions (pH = 3 and 50% CO_2), indicated that the industrial SA-producing strain *S. cerevisiae* SUC632 was more robust than the congenic laboratory strain CEN.PK113-7D, as the k_d of the industrial strain, was about threefold lower than that of the laboratory strain (Hakkaart et al., 2020). However, at near-zero growth rates in low-pH retentostat cultures, the k_d values of the two strains were the same (0.0030 ± 0.0004 and 0.0030 ± 0.0006 h^{-1}, respectively; Hakkaart et al., 2020).

With respect to the industrial application of the zero-growth concept, the use of membrane filters to achieve cell retention might be less feasible on an industrial scale, as blocking of effluent filters could prevent long-time operation. Alternative means of cell retention could involve the use of fast-sedimenting yeast mutants (Oud et al., 2013) or cell immobilization (Gulli et al., 2019; Nagarajan et al., 2014). Near-zero growth conditions can also be achieved by controlling the feed of a limiting nutrient in fed-batch processes. Due to the absence of a liquid outflow, fed-batch cultivation results in high final product titers, which are beneficial for the downstream processing. Wahl et al. (2017) studied growth of the same SA-producing *S. cerevisiae* strain in fed-batch cultures which, during the final phase of cultivation, were starved for ammonium. In contrast to our observations on nitrogen-limited retentostat cultures, the specific SA production rate observed after ammonium depletion was not stable but declined to a very low value within 90 h. As the authors expressed specific SA productivity per total amount of biomass and did not measure culture viability, this result may have reflected a loss of culture viability caused by the low cultivation pH (Hakkaart et al., 2020), complete nitrogen starvation and/or high SA concentrations. The SA yield on glucose in the N-starved fed-batch cultures declined from 0.64 ± 0.01 to 0.53 ± 0.01 mol SA per mol glucose during the final phase of fermentation (Wahl et al., 2017). In contrast, our ammonium-limited retentostat cultures the SA yield on glucose stabilized to a value of 0.60 mol SA per mol glucose (0.4 Cmol/Cmol), which was maintained until the end of the 3-week retentostat runs (Figure 3). The lower SA yield in the fed-batch cultures may be related to the much higher SA concentrations (up to 0.6 mol/L, as compared to 63 ± 3 mmol/L in the retentostat cultures). A high SA titer at a low cultivation pH will result in entry of undissociated SA across the plasma membrane by passive diffusion, followed by active export by the heterologous DCT02 transporter from *Aspergillus niger* (Jansen et al., 2013). Experiments with ^{13}C-SA demonstrated the relevance of such an ATP dissipating futile cycle, which necessitates an increased rate of glucose dissimilation to CO_2 and water and, consequently, causes a lower SA yield on glucose (Wahl et al., 2017).

Measurements of intracellular succinate and other TCA-cycle intermediates in N-limited retentostat cultures showed levels consistent with the genetic makeup of the industrial strain, whose metabolism has been rewired toward the reductive conversion of oxaloacetate to succinate in the yeast cytosol by high-level expression of native and heterologous enzymes (Jansen et al., 2013). Similar intracellular metabolite levels were reported for ammonium-starved fed-batch cultures of the same strain (Wahl et al., 2017). The very high mass action rations of the fumarase reaction that were observed in both studies indicate a strong driving force in the direction of fumarate synthesis and thus SA production. However, ^{13}C flux analysis in the fed-batch cultures showed that the oxidative TCA cycle flux was significantly higher than the reductive flux toward succinate. The net fumarase flux was therefore in the direction of malate, which would require an in vivo mass action ratio below K_{eq}. Wahl et al. (2017) explained the high value of the mass action ratio from subcellular compartmentation of malate. The high malate to fumarate ratio then suggests a possible limitation of cytosolic fumarase for use of the reductive pathway toward SA, which could potentially be relieved by modifying in vivo kinetic and/or regulatory characteristics of this key enzyme.

With this study, we have shown that long-term extreme limitation by a nutrient other than the carbon and energy source can enable stable conversion of substrate into product in the virtual absence of growth. Although we have used retentostat systems in this study, a similar concept could be applied in repeated fed-batch cultivations, in which the producing strain is used as a biocatalyst for prolonged periods of time and conversion of substrate into biomass is minimized. Further strain optimization should focus on improving glucose conversion rates at near-zero growth rates and, in particular, on increasing cell viability during prolonged cultivation at low pH.

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AUTHOR CONTRIBUTIONS

Yaya Liu and Osman Esen performed the chemostat experiments. Yaya Liu performed the retentostat experiments. Yaya Liu analyzed the samples and the data, and wrote the original draft of the manuscript. Walter M. van Gulik and Jack T. Pronk reviewed the manuscript. All authors reviewed, edited, and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available below: Liu, Yaya (2020): Research data for the Ph.D. thesis: "Uncoupling yeast growth and product formation in chemostat and retentostat cultures." 4TU.ResearchData. Dataset. https://doi.org/10.4121/uuid:65975daa-57bb-4f29-9f0a-657161ea1347.

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