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, i.e., at a culture pH of 3 and with sparging of a 1:1 CO2-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 biomass-specific SA production rate and glucose consumption rate indicated a 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*.

Introduction

Succinic acid (SA), a C4 dicarboxylic acid and intermediate of the tricarboxylic acid (TCA) cycle (Tretter, Patocs, & Chinopoulos, 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, Jain, & Elankovan, 1999). Based on predicted environmental benefits, 2004 and 2010 reports of the U.S. 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). In order to supply this market, bio-based production of SA will become increasingly important as it can provide a sustainable alternative to petrochemical production (Pinazo, Domine, Parvulescu, & Petru, 2015).

In recent years, production of SA in industrial fermentation processes has been realized with naturally SA-producing microorganisms as well as with engineered strains (Beauprez, De Mey, & Soetaert, 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 bovine rumen (Kuhnert, Scholten, Haefner, Mayor, & Frey, 2010). Myriant applied an engineered*E.coli* strain for large-scale SA production (Ahn, Jang, & Lee, 2016). However, SA-producing bacteria may be affected by bacteriophage infection 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, Zelle, Pronk, & van Maris, 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 infection, 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 *S. cerevisiae* strain for industrial scale SA production (Jansen, Heijnen, & Verwaal, 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, de Hulster, Kloezen, Pronk, & van Maris, 2010). Although the Gibbs free energy changing of the synthesis of SA from glucose is negative, the costs of active SA export (2 to 3 mol ATP per mol SA exported) (Taymaz-Nikerel et al., 2013) require 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, Bernal Martinez, Zhao, van Gulik, & Jansen, 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 labelled 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 rate. This observation was attributed to increased product degradation as well as to increased non-growth 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 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 steady state. As an alternative to chemostat cultivation, retentostat cultures have proven to be excellent tools to study growth of *S. cerevisiae* and other microorganisms at low to near-zero growth rates under various nutrient limitations (Boender, de Hulster, van Maris, Daran-Lapujade, & Pronk, 2009; Ercan et al., 2015; Hakkaart et al., 2020; Liu, El Masoudi, Pronk, & van Gulik, 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, i.e. 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 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 SA depends 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.

Materials and methodsYeast strain and growth media

The engineered SA-overproducing industrial *S. cerevisiae* strain (SUC632) was kindly provided by Royal DSM B.V (Delft, the Netherlands). 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, 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 $MgSO_4.7H_2O$, 1 ml trace-element solution and 1 ml vitamin solution (Verduyn, Postma, Scheffers, & Van Dijken, 1992). After filter sterilization (Millex® Syringe Filters, 0.22 μm, Merck Millipore, Massachusetts, USA), 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 $MgSO_4.7H_2O$, 1 ml trace element solution, 1 ml vitamin solution, 1 ml of a solution containing 3 g/L $FeSO_4.7H_2O$ and 15 g/L EDTA, 1 ml of biotin solution (1 g/L), and 0.2 g Pluronic 6100 PE antifoaming agent (BASF, Ludwigshafen, Germany). 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.

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, Melsungen, Germany).

Batch, chemostat and retentostat cultivations were carried out in 7 L bioreactors (Applikon, Delft, The Netherlands) 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, Schiedam, The Netherlands) 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, Greinfensee, Switzerland) 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, Overberg, The Netherlands). CO_2 and O_2 concentrations in the dried gas were measured with a Rosemount NGA 2000 gas analyzer (Minnesota, USA).

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 2 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, Delft, The Netherlands) mounted inside the reactor (Liu et al., 2019). The cell-free effluent was pumped in a sterile effluent vessel using a peristaltic pump (Masterflex, Barrington, USA) controlled by the vessel weight, such that the broth weight was kept at 5.00 ± 0.05 kg. During retentostat cultivation, 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.

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, Fullerton, CA). Cell viability of culture samples was determined with a Funga-Light 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).

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).

Quantification of substrates, products and intracellular metabolites

Ammonium concentrations were analysed with a Gally Discrete Analyzer (ThermoFisher Scientific, Massachusetts, United States) with a detection limit of 0.02 mg $NH4^+$/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).

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, 2000).

Results

Growth and viability of S. cerevisiae SUC632 in ammonium-limited chemostat and retentostat cultures at low pH and high CO_2levels

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, Boles, & Ciriacy, 1997), indicating that ammonium was indeed the growth limiting nutrient (Supporting Information Table S1).

In all chemostat cultures, viability was between 80 and 90 % (Figure 1A). As only the viable cell fraction contributes to substrate consumption, growth and (by)product formation, specific conversion rates were expressed per g 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 levelled 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 grown at 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}, meanwhile the corresponding specific growth rates (μ) also rapidly dropped and approximately stabilized at values below 0.005 h^{-1} (Figure 1H).

Uncoupling of growth and succinic acid production

In nitrogen-limited retentostat cultures, the biomass-specific SA production rate decreased asymptotically with decreasing specific growth rate and stabilized at a value of 0.75 mmol/(g viable biomass)/h for specific growth rates between 0.008 and 0.014 h^{-1}(Figure 2). 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 h. 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 Supporting Information Table S2.

The fraction of consumed glucose distributed to biomass, SA and byproducts formation were compared between those different specific growth rates cultures (Figure 3). Specifically, the fraction of the consumed glucose converted to SA was highest in the condition that growth was virtually absent (Figure 3). Besides, during retentostat cultivation, the yield of SA on glucose progressively decreased from almost 0.9 mol/mol until, after 400 h, it reached a stable value of approximately 0.6 mol/mol (Figure 4).

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 adenylate energy charge and ATP/ADP ratios were analysed (Figure 5). During retentostat cultivation, intracellular levels of ATP and ADP slowly decreased (Figure 5B and C) and stabilized after about 350 h. Retentostat cultures showed a stable intracellular AMP level that approximately two-fold higher than observed in the preceding chemostat culture (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 to 2.0 at the end of the retentostat cultivation (Figure 5E).

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). 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 during retentostat cultivation, 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, Ras, ten Pierick, van Gulik, & Heijnen, 2011) levels of citrate, succinate and malate were 5, 90 and 25 fold higher, respectively, towards 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 conversion of 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.

Discussion

In this study, we explored the feasibility of applying cultivation at near-zero growth rate for high-yield production of chemicals from renewable feedstocks. As a model system we used an engineered industrial strain of *S. cerevisiae* , capable of producing SA from glucose. To simulate industrial process conditions, growth studies were performed at low pH and at an elevated CO_2 concentration.

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 near-zero growth rate in retentostat cultures has been reported to result in accumulation of non-viable, non-producing cells (Boender et al., 2009; Vos et al., 2016). A previous study with the non-producing laboratory reference strain CEN.PK113-7D (Hakkaart et al., 2020) showed that, at pH 3, the specific death rates (k_d) in glucose- and ammonium-limited retentostat cultures (respectively 0.0039 ± 0.0005 and 0.0030 ± 0.0004 h^{-1}) were approximately eight-fold higher than in corresponding cultures grown at pH 5. However, cultivation of the reference strain at near-zero growth rate as such did not cause an increased k_d, since the death rates in glucose- and ammonium-limited retentostat cultures performed at pH 3 were two-fold and tenfold lower, respectively, than in corresponding chemostat cultures grown at a specific growth rate of 0.025 h^{-1} (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 3-fold lower than that of the laboratory strain (Hakkaart et al., 2020). At near-zero growth rates in low-pH retentostat cultures, k_d values of the two strains were not significantly different (0.0030 ± 0.0004 and 0.0030 ± 0.0006 h^{-1}, respectively) (Hakkaart et al., 2020).

In the ammonium-limited chemostat and retentostat cultures, biomass-specific rates of SA production decreased asymptotically with decreasing growth rate to an essentially constant value of 3 ± 0.1 mCmol/(g viable biomass)/h at specific growth rates below 0.02 h^{-1} (Figure 2B). This productivity was maintained until, after 525 h (three weeks) the retentostat cultures were terminated. These results demonstrated that cell retention in ammonium-limited cultures can result in sustained, stable SA production in the virtual absence of growth. The combined chemostat and retentostat data showed that biomass-specific SA production rates increased linearly with glucose consumption rate (Figure 7). This observation strongly suggests that 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 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.

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 use of fast-sedimenting yeast mutants (Oud et al., 2013) or immobilization (Gulli et al., 2019; Nagarajan et al., 2014) might be explored. 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 (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 observation 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 cause 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, which was maintained until the end of the three-week retentostat runs (Figure 4). 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 DCT02 transporter. Experiments with ^{13}C-SA demonstrated the relevance of such a 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 towards 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 towards succinate. The net fumarase flux was therefore in the direction of malate, which would require a *in vivo* mass action ratio below K_{eq}. Wahl et al. explained the high value of the mass action ratio from subcellular compartmentation of malate (Wahl et al., 2017). The high malate to fumarate ratio then suggests a possible limitation of cytosolic fumarase for use of the reductive pathway towards SA, which could potentially be relieved by modifying *in vivo* kinetic and/or regulatory characteristics of this key enzyme .

With this work we have shown that long-term extreme limitation by a nutrient other than the carbon- and energy source enable stable conversion of substrate into product in the virtual absence of growth. Although we have used retentostat systems in this research, 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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References

Abbott, D. A., Zelle, R. M., Pronk, J. T., & van Maris, A. J. (2009). Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. *FEMS Yeast Res, 9* (8), 1123-1136. doi:10.1111/j.1567-1364.2009.00537.x

Ahn, J. H., Jang, Y. S., & Lee, S. Y. (2016). Production of succinic acid by metabolically engineered microorganisms. *Curr Opin Biotechnol, 42* , 54-66. doi:10.1016/j.copbio.2016.02.034

Beauprez, J. J., De Mey, M., & Soetaert, W. K. (2010). Microbial succinic acid production: Natural versus metabolic engineered producers.*Process Biochem, 45* (7), 1103-1114. doi:10.1016/j.procbio.2010.03.035

Boender, L. G. M., de Hulster, E. A. F., van Maris, A. J. A., Daran-Lapujade, P. A. S., & Pronk, J. T. (2009). Quantitative Physiology of *Saccharomyces cerevisiae* at Near-Zero Specific Growth Rates. *Appl Environ Microbiol, 75* (17), 5607-5614. doi:10.1128/aem.00429-09

Boles, E., & Hollenberg, C. P. (1997). The molecular genetics of hexose transport in yeasts. *FEMS Microbiol Rev, 21* (1), 85-111. doi:10.1111/j.1574-6976.1997.tb00346.x

Bozell, J. J., & Petersen, G. R. (2010). Technology development for the production of biobased products from biorefinery carbohydrates - the US Department of Energy's "Top 10" revisited. *Green Chemistry, 12* (4). doi:10.1039/b922014c

Canelas, A. B., Ras, C., ten Pierick, A., van Gulik, W. M., & Heijnen, J. J. (2011). An *in vivo* data-driven framework for classification and quantification of enzyme kinetics and determination of apparent thermodynamic data. *Metab Eng, 13* (3), 294-306. doi:10.1016/j.ymben.2011.02.005

Chen, Y., & Nielsen, J. (2013). Advances in metabolic pathway and strain engineering paving the way for sustainable production of chemical building blocks. *Curr Opin Biotechnol, 24* (6), 965-972. doi:10.1016/j.copbio.2013.03.008

Cipollina, C., ten Pierick, A., Canelas, A. B., Seifar, R. M., van Maris, A. J., van Dam, J. C., & Heijnen, J. J. (2009). A comprehensive method for the quantification of the non-oxidative pentose phosphate pathway intermediates in *Saccharomyces cerevisiae* by GC-IDMS.*J Chromatogr B Analyt Technol Biomed Life Sci, 877* (27), 3231-3236. doi:10.1016/j.jchromb.2009.07.019

Dessie, W., Xin, F., Zhang, W., Jiang, Y., Wu, H., Ma, J., & Jiang, M. (2018). Opportunities, challenges, and future perspectives of succinic acid production by *Actinobacillus succinogenes* . *Appl Microbiol Biotechnol, 102* (23), 9893-9910. doi:10.1007/s00253-018-9379-5

Ercan, O., Bisschops, M. M., Overkamp, W., Jorgensen, T. R., Ram, A. F., Smid, E. J., . . . Kleerebezem, M. (2015). Physiological and Transcriptional Responses of Different Industrial Microbes at Near-Zero Specific Growth Rates. *Appl Environ Microbiol, 81* (17), 5662-5670. doi:10.1128/AEM.00944-15

Gulli, J., Cook, E., Kroll, E., Rosebrock, A., Caudy, A., & Rosenzweig, F. (2019). Diverse conditions support near-zero growth in yeast: Implications for the study of cell lifespan. *Microb Cell, 6* (9), 397-413. doi:10.15698/mic2019.09.690

Hakkaart, X., Liu, Y., Hulst, M., el Masoudi, A., Peuscher, E., Pronk, J., . . . Daran-Lapujade, P. (2020). Physiological responses of*Saccharomyces cerevisiae* to industrially relevant conditions: slow growth, low pH and high CO_2 levels.*Biotechnol Bioeng, 117* (3), 721-735. doi:10.1002/bit.27210

Hewitt, C. J., & Nienow, A. W. (2007). The Scale-Up of Microbial Batch and Fed-Batch Fermentation Processes. *Adv Appl Microbiol, 62* , 105-135. doi:10.1016/s0065-2164(07)62005-x

Jamalzadeh, E. (2013). *Transport of Dicarboxylic Acids in Saccharomyces cerevisiae.* (Ph. D.), Delft University of Technology, Delft.

Jansen, M. L., Heijnen, J. J., & Verwaal, R. (2013). The Netherlands Patent No. WO 2013/004670 Al.

Jansen, M. L., & van Gulik, W. M. (2014). Towards large scale fermentative production of succinic acid. *Curr Opin Biotechnol, 30* , 190-197. doi:10.1016/j.copbio.2014.07.003

Kuhnert, P., Scholten, E., Haefner, S., Mayor, D., & Frey, J. (2010).*Basfia succiniciproducens* gen. nov., sp. nov., a new member of the family *Pasteurellaceae* isolated from bovine rumen. *Int J Syst Evol Microbiol, 60* (Pt 1), 44-50. doi:10.1099/ijs.0.011809-0

Liu, Y., El Masoudi, A., Pronk, J. T., & van Gulik, W. M. (2019). Quantitative Physiology of Non-Energy-Limited Retentostat Cultures of*Saccharomyces cerevisiae* at Near-Zero Specific Growth Rates.*Appl Environ Microbiol, 85* (20), e01161-01119. doi:10.1128/AEM.01161-19

Luthfi, A. A. I., Manaf, S. F. A., Illias, R. M., Harun, S., Mohammad, A. W., & Jahim, J. M. (2017). Biotechnological route for sustainable succinate production utilizing oil palm frond and kenaf as potential carbon sources. *Appl Microbiol Biotechnol, 101* (8), 3055-3075. doi:10.1007/s00253-017-8210-z

Nagarajan, S., Kruckeberg, A. L., Schmidt, K. H., Kroll, E., Hamilton, M., McInnerney, K., . . . Rosenzweig, F. (2014). Uncoupling reproduction from metabolism extends chronological lifespan in yeast. *PNAS* , 201323918. doi:10.1073/pnas.1323918111

Oud, B., Guadalupe-Medina, V., Nijkamp, J. F., de Ridder, D., Pronk, J. T., van Maris, A. J. A., & Daran, J.-M. (2013). Genome duplication and mutations in *ACE2* cause multicellular, fast-sedimenting phenotypes in evolved *Saccharomyces cerevisiae* . *PNAS, 110* (45), E4223-E4231. doi:10.1073/pnas.1305949110

Pinazo, J. M., Domine, M. E., Parvulescu, V., & Petru, F. (2015). Sustainability metrics for succinic acid production: A comparison between biomass-based and petrochemical routes. *Catalysis Today, 239* , 17-24. doi:10.1016/j.cattod.2014.05.035

Reifenberger, E., Boles, E., & Ciriacy, M. (1997). Kinetic Characterization of Individual Hexose Transporters of*Saccharomyces Cerevisiae* and their Relation to the Triggering Mechanisms of Glucose Repression. *Eur J Biochem, 245* (2), 324-333. doi:10.1111/j.1432-1033.1997.00324.x

Seifar, R. M., Ras, C., van Dam, J. C., van Gulik, W. M., Heijnen, J. J., & van Winden, W. A. (2009). Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry.*Anal Biochem, 388* (2), 213-219. doi:10.1016/j.ab.2009.02.025

Shah, M. V. (2016). *Dicarboxylic acids transport, metabolism and production in aerobic Saccharomyces cerevisiae.* (P.h. D), Delft University of Technology, Delft.

Taymaz-Nikerel, H., Jamalzadeh, E., Borujeni, A. E., Verheijen, P. J. T., van Gulik, W. M., & Heijnen, J. J. (2013). A thermodynamic analysis of dicarboxylic acid production in microorganisms. In U. von Stockar & L. A. M. van der Wielen (Eds.), *The Role of Thermodynamics in Biochemical Engineering* (pp. 547-579). New York.

Tretter, L., Patocs, A., & Chinopoulos, C. (2016). Succinate, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. *Biochim Biophys Acta, 1857* (8), 1086-1101. doi:10.1016/j.bbabio.2016.03.012

van Dam, J. C., Eman, M. R., Frank, J., Lange, H. C., van Dedem, G. W. K., & Heijnen, S. J. (2002). Analysis of glycolytic intermediates in*Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionization with tandem mass spectrometric detection.*Analytica Chimica Acta, 460* (2), 209-218. doi:10.1016/S0003-2670(02)00240-4

Verduyn, C., Postma, E., Scheffers, W. A., & Van Dijken, J. P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast, 8* (7), 501-517. doi:10.1002/yea.320080703

Verheijen, P. J. T. (2000). *Data reconciliation and error detection* : Boca Raton: CRC Press.

Vos, T., Hakkaart, X. D., de Hulster, E. A., van Maris, A. J., Pronk, J. T., & Daran-Lapujade, P. (2016). Maintenance-energy requirements and robustness of *Saccharomyces cerevisiae* at aerobic near-zero specific growth rates. *Microb Cell Fact, 15* (1), 111. doi:10.1186/s12934-016-0501-z

Wahl, S. A., Bernal Martinez, C., Zhao, Z., van Gulik, W. M., & Jansen, M. L. A. (2017). Intracellular product recycling in high succinic acid producing yeast at low pH. *Microb Cell Fact, 16* (1), 90. doi:10.1186/s12934-017-0702-0

Werpy, T., & Petersen, G. (2004). *Top Value Added Chemicals from Biomass: Volume I - Results of Screening for Potential Candidates from Sugars and Synthesis Gas* . Retrieved from https://www.osti.gov/servlets/purl/15008859

Wu, L., Mashego, M. R., van Dam, J. C., Proell, A. M., Vinke, J. L., Ras, C., . . . Heijnen, J. J. (2005). Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly 13C-labeled cell extracts as internal standards.*Analytical Biochemistry, 336* (2), 164-171. doi:10.1016/j.ab.2004.09.001

Zeikus, J. G., Jain, M. K., & Elankovan, P. (1999). Biotechnology of succinic acid production and markets for derived industrial products.*Appl Microbiol Biotechnol, 51* (5), 545-552. doi:10.1007/s002530051431

Zelle, R. M., de Hulster, E., Kloezen, W., Pronk, J. T., & van Maris, A. J. A. (2010). Key Process Conditions for Production of C_4 Dicarboxylic Acids in Bioreactor Batch Cultures of an Engineered *Saccharomyces cerevisiae* . *Appl Environ Microbiol, 76* (3), 744-750. doi:10.1128/AEM.02396-09

Figure legends

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 *S. cerevisiae* SUC632. Cultures were grown on glucose, at pH 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.

Figure 2 Biomass specific consumption rates of glucose and production rates of succinic acid and by-products as a function of the specific growth rate (μ) in aerobic, ammonium-limited chemostat and retentostat cultures of *S. cerevisiae* SUC632. Cultures were grown on glucose, at pH 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. All data represent averages of results from duplicate chemostat and retentostat cultures with their standard errors.

Figure 3 Growth rate dependent division of consumed substrate between growth, succinic acid and byproducts formation in aerobic, ammonium-limited chemostat and retentostat cultures of *S. cerevisiae* SUC632, at different specific growth rates. Cultures were grown on glucose, at pH 3 and were sparged with a 1:1 mixture of air and CO_2.

Figure 4 Succinic acid yield on glucose during ammonium-limited aerobic retentostat cultivation. Retentostat cultures were grown on glucose, at pH 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.

Figure 5 Intracellular adenosine phosphate level, adenylate energy charge and ATP/ADP ratio during aerobic, ammonium limited retentostat cultivation of *S. cerevisiae* SUC632. Retentostat cultures were grown on glucose, at pH 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. Data represent the averages and standard errors of measurements from duplicate cultures.

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 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. Dashed blue lines in Figures G and H represent the apparent *in vivo* equilibrium constants determined for*S. cerevisiae* (Canelas et al., 2011). Data represent the averages and standard errors of measurements from duplicate cultures.

Figure 7 Specific succinic acid production rate plotted as a function of specific glucose consumption rate in aerobic, ammonium-limited chemostat and retentostat cultures of *S. cerevisiae* SUC632. Cultures were grown on glucose, at pH 3 and were sparged with a 1:1 mixture of air and CO_2. All data represent averages with standard errors obtained from duplicate chemostat and retentostat cultivations.

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