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Exploring Yeast's Energy Dynamics: The General Stress Response Lowers Maintenance Energy Requirement

Nuran Temelli | Simon van den Akker | Ruud A. Weusthuis | Markus M. M. Bisschops

Bioprocess Engineering, Wageningen University, Wageningen, the Netherlands

Correspondence: Markus M. M. Bisschops (mark.bisschops@wur.nl)

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ABSTRACT

In many microbial biotechnology processes, biomass itself is not the product of interest, but rather targeted chemicals or proteins. In these processes, growth should be limited to direct more substrate to product and increase process yields. Under growth-limiting conditions, such as nutrient limitation, microorganisms, including the yeast *Saccharomyces cerevisiae*, activate a general stress response (GSR). Different hypotheses have been formulated for this activation, including a preparatory role for future stresses or a role in cellular protein density. Here we tested a third hypothesis: the GSR reduces the energy needed to maintain cellular homeostasis, also known as the maintenance energy requirement (MER). The impact of GSR on MER was investigated by assessing the effect of the absence of its key regulators, Msn2 and Msn4, on energy-substrate distribution and stress resistance. Chemostat and fed-batch cultures revealed significant increases in MER of up to 85% in the deletion strain compared to the parental strain. In contrast, maximal biomass yields, growth rates and morphology were unaffected. Our insights highlight an additional role of the GSR, namely saving cellular energy. As the MER is a key determinant of product yields and in process design, especially in low growth processes, our findings can help to optimise microbial bioprocesses.

1 | Introduction

Metabolic energy conserved in dissimilation is used for three competing processes: growth, maintenance and—if applicable—product formation. Energy investment in growth is desired if biomass, for example, single-cell protein, is the product of interest. In most cases, however, biomass is not the desired product and energy directed towards growth comes with concomitant substrate loss and reduced product yields. Cells also need energy to maintain cellular homeostasis (Van Bodegom 2007). This amount of energy is known as the maintenance energy requirement (MER), and its impact on bioprocesses is multifaceted.

The MER must be included in the cultivation design to supply cells with sufficient substrate to remain alive and productive. As such, it is an especially important factor in energy- and oxygen-limited cultivations, such as widely applied fed-batch cultures. Too low feeding rates of the energy substrate—such as glucose—or too low oxygen-transfer rates in high cell density cultures result in insufficient ATP generation through respiration, eventually leading to cell death. The value of the MER impacts process economics differently for catabolic products, such as ethanol, as opposed to energy-requiring products, such as recombinant proteins. A high MER is beneficial in the case of catabolic products, as it will increase product yields and reduce biomass formation. In contrast, energy-requiring products compete for cellular energy with maintenance, and a low MER allows for higher product yields. The impact is especially strong under slow-growing conditions, where a more significant fraction of the energy substrate is used for maintenance. For example, in aerobic slow-growing yeast cultures ($\mu = 0.03\,h^{-1}$), 27% of the glucose consumed is used for

the maintenance of cellular homeostasis, while the glucose fraction needed at a 0.15 h^{-1} specific growth rate is only 5% (Vos et al. 2015). Optimization of the MER thus allows more substrate to be converted into the desired product (Russell and Cook 1995). Especially for energy-requiring products, there is considerable potential to increase production efficiency by reducing the MER.

To estimate the MER of a microorganism under specific conditions, the models as postulated by Pirt and Herbert to describe the division of substrate between growth and maintenance can be used (Herbert et al. 1956; Pirt 1982). These models are summarised in Equation (1), which describes a linear correlation between the specific substrate consumption rate (q_S) and specific growth rate (μ).

$$q_s = \frac{\mu}{Y_{XS}^{max}} + m_S \quad (1)$$

Besides the specific substrate consumption rate, this equation also contains the maximum theoretical yield ($Y_{XS}{}^{max}$) of biomass (X) on the substrate (S) and the maintenance coefficient (m_S) needed to fulfil the MER. To find these parameters, q_S values at various specific growth rates need to be determined, for which chemostat cultures at various dilution rates are often used (Ercan et al. 2015). Linear regression then allows the estimation of both parameters. However, the measurements are often taken at relatively high specific growth rates, where most of the sugar is consumed for biomass formation, and the portion for m_S is relatively low (< 10%). Although this allows accurate estimation of $Y_{XS}{}^{max}$, it also makes the value estimated for m_S sensitive to minor deviations in measurements, as shown earlier (Ercan et al. 2015; Vos et al. 2015). Conversely, glucose-limited retentostat cultures gradually reach near-zero growth rates, allowing a more accurate MER estimation. Retentostats are, however, labour-intensive and time-consuming. It takes several weeks to reach near-zero growth rates (Boender et al. 2009; Bisschops et al. 2014; Ercan et al. 2015; Vos et al. 2016) and are, therefore, less often used.

The few studies that employed energy-limited retentostats to culture microorganisms, including the yeasts *S. cerevisiae*, *K. phaffii* and *O. parapolymorpha* (Bisschops et al. 2014; Rebnegger et al. 2016; Juergens et al. 2024) showed that this cultivation mode can be used to reach a virtual maintenance-only state, and MER values were estimated. More in-depth omics analysis revealed that, as expected, most biosynthetic processes were down-regulated at near-zero growth rates. However, levels of many gene transcripts and proteins did not significantly change, and some increased (Boender et al. 2011; Binai et al. 2014; Vos et al. 2016). Some of these are responsible for processes conventionally classified as maintenance processes, such as ion homeostasis. Examples are Hsp30, which controls plasma membrane ATPase activity (Piper et al. 1997; Boender et al. 2011; Binai et al. 2014), and protein turnover by autophagy. Perhaps more intriguing is the increased expression of genes involved in stress resistance; a shared response observed in slow to nongrowing microbes of different kingdoms (Ercan et al. 2015). This increased expression also translates into a more stress-resistant phenotype (Boender et al. 2011; Bisschops et al. 2014; Bisschops et al. 2015; Vos et al. 2016).

This increased expression is part of the so-called general stress response (GSR). The GSR is a common response to diverse adverse conditions that aids cells in surviving those stressors. In other words, even in the presence of a single stressor, cells increase their resistance against multiple potential stresses (Gasch et al. 2000). Several transcription factors are involved in yeast stress responses, but most of these are rather stress specific. For example, Heat shock factor 1 is merely involved in heat and proteotoxic stress, Skn7 in oxidative stress and Gis1 is activated during the diauxic shift in *S. cerevisiae* (Morgan et al. 1997; Orzechowski Westholm et al. 2012; Joutsen and Sistonen 2019). However, the transcription factors Msn2 and Msn4 are well documented for their role in regulating GSR under various stress conditions, including (extreme) nutrient limitation (Gasch et al. 2000; Bisschops et al. 2014; Vos et al. 2016; Kunkel et al. 2019). These general stress regulators are also present in other industrially relevant yeasts, including *K. phaffii, O. polymorpha* and *Y. lipolytica* (Pomraning et al. 2018; Seike et al. 2021; Bernat-Camps et al. 2023). The cellular localization and thereby activity of Msn2 and Msn4 are controlled by the PKA and TORC1 nutrient-signalling pathways (Smets et al. 2010; Kunkel et al. 2019). Nutrient depletion or stress leads to PKA and TORC1 inhibition, which results in Msn2/4p's translocation from the cytoplasm to the nucleus. In the nucleus, Msn2/4p binds to Stress Response Elements (STRE) in promoters of around 600 genes and activates their expression (Boy-Marcotte et al. 1998; Gasch et al. 2000; Durchschlag et al. 2004; Sweeney and McClean 2023).

The GSR allows cells to adapt to suboptimal environments and regulates several processes involved in maintaining cellular homeostasis. Examples of processes impacted by the GSR are storage carbohydrate metabolism, redox-cofactor balancing, protein (re)folding and degradation, DNA repair and intracellular signalling (Gasch et al. 2000). Why these proteins are higher expressed in virtually non-dividing cells is still debated. Two common, and not per se mutually exclusive, explanations are linked to preparatory behaviour and cellular (protein) constraints. One hypothesis is that GSR helps prepare cells for deteriorating environmental conditions (Binai et al. 2014). Cross-resistance triggered by a specific stress, such as nutrient limitation or starvation, can offer a selective advantage in environments where multiple stresses regularly coincide or succeed each other. Under those conditions, not enough resources, e.g., nutrients or biosynthetic capacity, might be available to respond to the second stress and cross-resistance hence offers an advantage at the population level. Another reasoning is built on the concept of maximising fitness, or maximal reproduction under given conditions, within cellular constraints (Bruggeman et al. 2020). This implies that under optimal conditions, much of the cell's resources are dedicated to biosynthesis, such as ribosomes, to maximise growth. Proteins that do not directly contribute to growth, such as those involved in stress resistance, result in a burden and reduced fitness (Nyström 2004; Bruggeman et al. 2020). Under these conditions, growth can be considered limited by the intracellular, cytosolic protein availability. Under suboptimal conditions, other constraints, such as the membrane protein capacity, result in a reduced growth rate. Under these conditions, growth is no longer constrained by the cytosolic protein capacity, which gives room for other proteins, including those

TABLE 1 | *S. cerevisiae* strains used in this study.

Species	Strain	Relevant genotype	Reference
S. cerevisiae	CEN.PK113-7D	Haploid, prototrophic reference strain	Nijkamp et al. (2012)
S. cerevisiae	ScNtd002	CEN.PK113-7D Δ*msn2*Δ*msn4*	This study
S. cerevisiae	CEN.PK113-7D-*pHSP12-mRuby2*	CEN.PK113-7D X-2::*pHSP12- mRuby2-ADH1t*	This study
S. cerevisiae	ScNtd002- *pHSP12-mRuby2*	CEN.PK113-7D X-2::*pHSP12-mRuby2-ADH1t* Δ*msn2*Δ*msn4*	This study

conferring stress resistance. Why the left-over space is filled with, at first glance, unneeded and costly proteins can be explained by an optimal protein density that maximises growth rate (Vazquez 2012). However, this does imply that there is an advantage in activating the general stress response over the mere expression of enzymes involved in biosynthesis. This advantage could be the stress resistance towards (further) deteriorating conditions, i.e., linked to the aforementioned hypothesis, and/or to maintain a certain saturation level of biosynthetic enzymes, e.g., ribosomes (Bosdriesz et al. 2015).

Here, we propose an additional hypothesis that explains the evolutionary driving force behind the observations described by the aforementioned theories. Several of the mechanisms activated by the general stress response may also have a role in energy saving. Heat-shock protein chaperones, for example, stabilise proteins and aid in the refolding of misfolded proteins (Clare and Saibil 2013), reducing costly protein turnover (Clare and Saibil 2013; Lahtvee et al. 2014). We, therefore, propose that GSR activation results in energy saving. As such, GSR activation would offer a direct advantage to cells, as more of the limiting energy resources can be dedicated to processes increasing fitness, being either more growth or prolonged survival.

2 | Experimental Procedures

2.1 | Strains and Strain Modification

The prototrophic strain *S. cerevisiae* CEN.PK113-7D (MATα, MAL2-8c, SUC2) was used in this study as well as a derived mutant that lacks the *MSN2* and *MSN4* genes (Table 1). To construct these mutants, yeast transformations were carried out using the lithium acetate method (Gietz and Schiestl 2007). Clones were selected on YPD with 200 μg/mL hygromycin. CRISPR-Cas9 was used for genomic deletions and integrations, based on the pUDP002 method developed by Juergens et al. (2018). This method introduces a single plasmid containing the *cas9* gene and selection markers, as well as gRNA sequences targeting the gene or region of interest. gRNA sequences used are shown in supplementary Table S1. Repair fragments for homologous recombination mediated deletion were designed according to the method described by Mans et al. (2015) (Table S2). The expression cassette consisting of the mRuby2 fluorescent protein coding sequence under the control of the *HSP12* promoter was constructed using the Yeast tool kit (Lee et al. 2015). The mRuby2 coding and *ADH1* terminator sequences were derived from part plasmids present in the yeast toolkit, while for the *HSP12* promoter a novel part plasmid was constructed. To this end, the 1000 bp upstream sequence of the *HSP12* start codon was amplified by PCR from CEN.PK113-7D genomic DNA and via a Golden Gate reaction assembled into the yeast toolkit entry vector as a part 2 plasmid using the Yeast tool kit instructions (Lee et al. 2015). The final expression cassette flanked by homologous arms (see Table S2) was integrated into the genome of the reference (CEN.PK113-7D) and double deletion Δ*msn2*Δ*msn4* (ScNtd002) strains at the previously characterised X-2 location (Mikkelsen et al. 2012). Successful integration or deletion was verified via colony PCR. The plasmids were cured by culturing the strains in YPD without selective pressure. Verified strains were grown in YPD until the late exponential phase and stored as glycerol stocks (30% v/v) at −80°C. All used primers and plasmids are shown in Tables S3 and S4, respectively.

2.2 | Shake Flask Cultivation

Cultures for stress assays were grown in 250 mL shake flasks containing 50 mL of yeast nitrogen base (YNB) medium (Sigma-Aldrich). The medium was prepared according to the manufacturer's instructions. It was supplemented with 20 g/L D-glucose, and the pH was adjusted to 6.0 using 2 M potassium hydroxide prior to filter sterilisation. The starting optical density (OD600) of the cultures was 0.15. Cultures for maximal specific growth rate (μ_{max}) determination were grown in 250 mL shake flasks containing 50 mL of synthetic medium (Verduyn et al. 1992). The pH was adjusted to 6.0 with 2 M potassium hydroxide before autoclaving. After autoclaving, it was supplemented with vitamins (Verduyn et al. 1992) and 20 g/L D-glucose. Cultures were inoculated at an OD of 0.15, and OD was measured at 600 nm in determined time intervals using a Hach Lange DR600 spectrophotometer. Precultures for shake flask experiments were prepared using the same medium, inoculated with 2 mL of frozen stock culture and grown overnight. Chemostat and fed-batch precultures were similarly inoculated with 2 mL of frozen stock cultures and grown in 500 mL shake flasks containing 100 mL of medium containing 20 g/L D-glucose. All shake flasks were incubated in an orbital shaker at 250 rpm at 30°C.

2.3 | Plate Reader Cultivation

Cultures were grown in 200 μL YNB or SMA medium supplemented with 20 g/L glucose in a black 96-well plate (Nunc, Thermo Fisher) with an initial OD_{600} of 0.15. Precultures were grown in 50 mL YNB overnight from −80°C glycerol stock in 250 mL shake flasks at 30°C with continuous shaking. The Synergy H1 plate reader (Biotek) measured the optical density

at 600 nm (OD_{600}) and mRuby2 fluorescent protein intensity (558 nm excitation, 605 nm emission) at 30°C, in orbital shaking at 5-min intervals. mRuby2 fluorescent protein intensity was normalised by dividing by the OD_{600} measurement at each time point.

2.4 | Chemostat and Fed-Batch Cultivations

Chemostat and fed-batch cultivations were performed in 3-l bioreactors (Applikon, Delft, the Netherlands). The operational parameters were set as follows: the temperature was maintained at 30°C, the constant stirring speed was 800 rpm, and pH was controlled at 5.0 using the automatic addition of 2 M potassium hydroxide. The cultures for the chemostat were grown in synthetic medium with 7.5 g/L glucose, 0.5 mL/L antifoam B (Sigma Aldrich), trace elements and vitamins (Verduyn et al. 1992). The cultures for the fed-batch were grown in YNB with 2.5 g/L ammonium sulfate, 0.5 mL/L antifoam B (Sigma-Aldrich) and 17 g/L glucose for the batch phase. The feed medium consisted of 70 g/L glucose, 13 g/L ammonium sulfate and 1.7 g/L dropout supplement (Sigma-Aldrich). In both chemostats and fed-batch cultivations, glucose was the sole carbon and energy source and the limiting nutrient. For the fed-batch cultivations, feeding started once the initial carbon source was exhausted during the batch phase, with a consistent feeding regime of 30 mL/L maintained throughout the experiment. The dilution rates for the chemostat cultivations were set at 0.025, 0.05, 0.1 and 0.2 h^{-1}, controlled by adjusting the medium inflow rate. Chemostat bioreactors were equipped with a level sensor to maintain a constant working volume of 1.4 L. Steady-state conditions were considered achieved after a minimum of five volume changes, verified by the stability of culture dry weight and specific carbon dioxide production rates, which varied by less than 5% over two consecutive volume changes.

2.5 | Determination of Cell Morphology and Viability

Samples were diluted to 1×10^7 with PBS buffer pH 7.5 (VWR) and incubated at room temperature for 15 min in the dark for viability tests. Both stained with propidium iodide (PI) and unstained samples were analysed on a Cytoflex flow cytometer (Beckman Coulter, the Netherlands), excited with 488 nm and measured with a 690 nm filter. A total of 10,000 cells were counted for each sample. To check for changes in cell morphology, forward and side scatter were measured as indicators of cell size and granularity, respectively.

2.6 | Stress Assays

To assess heat stress resistance, cells were grown in shake flasks on YNB media at 30°C. Mid-exponential or stationary phase culture samples were centrifuged at 5000 *g* for 3 min, resuspended in preheated 53°C PBS buffer pH 7.5 (VWR), and incubated in a 53°C water bath. Samples were collected at specified time intervals and immediately chilled on ice. For oxidative stress resistance determination, specified amounts of hydrogen peroxide were added to achieve final concentrations of 50 mM, 75 mM and 100 mM into the cultures in mid-exponential and stationary phases. These cultures were then incubated in an orbital shaker at 250 rpm, 30°C for 2 h. After incubation, the samples were centrifuged (5000 g for 3 min), washed with PBS, and resuspended in fresh PBS. The viability of all samples was determined using propidium iodide (PI) staining based on a previously described method (Boender et al. 2011). 4 μg/L PI stain was added to the prepared samples, which were then incubated for 15 min at room temperature in the dark. After incubation, samples were centrifuged (5000 g, 3 min) and resuspended in PBS. The treated samples were analysed using a Cytoflex flow cytometer (Beckman Coulter) equipped with a 488 nm excitation laser and a 690 nm bandpass filter.

2.7 | Determination of Substrate, Metabolite and Biomass Concentrations

The culture dry weight was determined by filtering precisely 10 mL of an appropriately diluted culture broth through pre-dried and pre-weighed membrane filters (pore size 0.45 μm, Gelman Science). Samples were diluted with demineralized water when the biomass concentration was higher than 15 g/L, prior to culture dry weight assays. The filters were then washed with demineralized water, dried for 20 min at 350 W in a microwave oven and reweighed (Postma et al. 1989). OD was measured at 600 nm using a Hach Lange DR600 spectrophotometer. Supernatants for HPLC analysis were prepared by centrifuging culture samples for 5 min at 16,000 × g. These supernatants were subsequently analysed using a high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity). This system was equipped with Rezex ROA-Organic acid H^+ (8%) 300 × 7.8 mm and operated at a flow rate of 0.8 mL/min at 60°C using 5 mM H_2SO_4 as the mobile phase. Detection was conducted using a dual-wavelength absorbance detector (Agilent G1314A) and a refractive-index detector (Agilent G1362A).

2.8 | Calculation of Fed-Batch Growth Kinetics

Specific substrate consumption rates (in g substrate per g biomass per hour) for fed-batch cultivation were calculated using Equation (2). In this equation, q_S denotes the specific substrate consumption rate (g/g/h) at time t, ϕ_L is the flow rate ($L \cdot h^{-1}$), $C_{S,\text{in}}$ is the glucose concentration in the feed (g/L), V_L(t) volume of the reactor (L) at time *t* and C_X(t) is the biomass concentration (g/L) at time *t*.

$$q_S(t) = \frac{\Phi_L \bullet C_{S,in}}{V_L(t) \bullet C_X(t)} \tag{2}$$

Biomass accumulation at time point *t* in fed-batch cultures was calculated with Equation (3), where μ is the specific growth rate (h^{-1}). This equation is derived from the biomass mass balance.

$$C_X(t_2) = \left(\frac{V_L(t_1) \bullet C_X(t_1)}{V_L(t_2)} \right) \bullet e^{(\mu \bullet (t_2 - t_1))} \tag{3}$$

The specific growth rate can be described by the Herbert–Pirt Equation (4), including glucose consumption for maintenance, in which Y_{XS}^{max} is the maximal yield of biomass on substrate ($g \cdot g^{-1}$) and m_S is the maintenance coefficient ($g \cdot g^{-1} \cdot h^{-1}$).

$$\mu = Y_{XS}^{max} \bullet \left(q_S - m_S\right) \tag{4}$$

Volume change during the run is calculated using Equation (2), where ϕ_L ($L \cdot h^{-1}$) is the flow rate, t (h) time and $V_{L,\text{in}}$ (L) is the initial reactor volume.

$$V_L = V_{L,in} + \phi_L \bullet t \tag{5}$$

The MER (m_S) and maximal biomass yields on substrate (Y_{XS}^{max}) ($g \cdot g^{-1}$) of *S. cerevisiae* CEN.PK113-7D and ScNtd002 in fed-batch cultures were estimated using minimisation of the sum of squared errors (SSE) between the measured biomass concentrations and predicted biomass concentrations, using the Excel solver function. The maximal value for Y_{XS}^{max} was constrained to 0.51 g/g.

The MER (m_S) and maximal biomass yields on substrate (Y_{XS}^{max}) of *S. cerevisiae* CEN.PK113-7D and ScNtd002 in chemostat cultures were estimated using linear regression analysis of q_S versus μ at the performed dilution rates (0.025, 0.05, 0.1 and 0.2 h^{-1}) in GraphPad Prism (GraphPad software LLC).

Statistical analysis of chemostat and fed-batch cultivations for parental and mutant strains was performed using linear regression. The analyses were conducted to assess whether there was a significant difference between the two experiments. Differences between the experiments were considered statistically significant if the p-value was less than 0.05. All calculations and regression analyses were performed in GraphPad Prism (GraphPad software LLC).

3 | Results

3.1 | Reduction in GSR by Deletion of *MSN2* and *MSN4*

To study the impact of the GSR on cellular energetics, we reduced the GSR by deletion of the genes *MSN2* and *MSN4* that encode the main transcriptional regulators of the GSR in yeast (Martínez-Pastor et al. 1996). To evaluate to what extent deletion of *MSN2* and *MSN4* reduces GSR, the survival of exponentially growing and stationary phase cells of both parental and deletion strains was compared upon exposure to heat shock and oxidative stress. The Δ*msn2*Δ*msn4* mutant (ScNTd002) showed reduced resistance to both stress conditions compared to the parental strain CEN.PK113-7D (Figure 1). Exponentially growing cells of both strains were highly sensitive to the applied stresses, but the Δ*msn2*Δ*msn4* mutant (ScNTd002) lost viability significantly faster ($p < 0.0001$) upon exposure to 53°C or 100 mM hydrogen peroxide (Figure 1A,C). As GSR has been reported to be activated during and after the diauxic shift (Boy-Marcotte et al. 1998; Gasch et al. 2000; Virgilio 2012), we expected a strong impact of the absence of Msn2 and Msn4 in stationary phase cultures. However, stationary phase cultures of the mutant and parental strains did not significantly differ in survival under the applied stresses (Figure 1B,D). This somewhat surprising observation might be explained by the redundant regulation of GSR. Other stress response transcription factors such as Yak1, Gis1 and Hsf1 may have taken over the activation of post-diauxic stress resistance. This activation of other transcription factors may have been strengthened by the complete absence of carbon source, reduced pH and possible oxygen limitation that occurs in the stationary phase shake flask cultures.

To further monitor the activation of the stress response, we used a biosensor consisting of the *HSP12* promoter driving the expression of the red fluorescent protein mRuby2 (Karreman and Lindsey 2005). mRuby2 expression was already observed in the early exponential phase for the reference strain on two different defined media (Table S5) and remained present throughout the entire batch cultivation. In contrast, virtually no fluorescence was observed in the Δ*msn2*Δ*msn4* strain during exponential growth and a minor increase during the post-diauxic shift and stationary phases (Figure S1). These activation patterns are in line with the results of the stress assays, where the strongest effect of the *MSN2* and *MSN4* deletion is observed in exponentially growing cultures.

The maximal specific growth rate (μ^{max}) is an indicator of microbial performance. To understand how the combined deletion of *MSN2* and *MSN4* affects the maximal flux capabilities, we compared the μ^{max} of both strains on glucose under non-stressed conditions. Both the parental and deletion strains grew with the same maximal specific growth rate (Figure 1E). Furthermore, the strains achieved similar optical densities (OD), indicative of similar biomass yields.

3.2 | GSR Does Not Impact Maximal Biomass Yield

To understand the impact of the GSR and, more specifically, the transcription factors Msn2 and Msn4 on carbon and energy distribution, we characterised the parental strain and mutant strain in chemostat cultures at different dilution rates (0.025, 0.05, 0.1 and 0.2 h^{-1}). Previous studies employing the same *S. cerevisiae* parental strain suggested that the correlation between the specific glucose uptake rate (q_S) and the growth rate (μ, equal to the dilution rate) is linear under a wide range of growth rates, both in the presence and absence of oxygen (Boender et al. 2009; Vos et al. 2016). This implied that the Pirt model (Equation (1)) holds and the glucose requirement for maintenance (m_S) is growth-rate independent. Indeed, a linear correlation between q_S and μ was observed for both the parental strain ($R^2 = 0.9960$, p-value < 0.0001) and the mutant strain ($R^2 = 0.9943$, p-value < 0.0001) at these growth rates (Figure 2a).

Using linear regression, the maximal theoretical biomass yields (corresponding to the inverse of the slope) of the parental strain CEN.PK113-7D and GSR reduced strain ScNtd001 were determined at 0.52 (±0.028) and 0.49 (±0.043) g/g, respectively (Figure 2b). This value is similar to experimental literature data for the same species, 0.51 and 0.50 g/g respectively in Verduyn et al. (1991) and Hoek et al. (2000). According to these results, the absence of Msn2 and Msn4

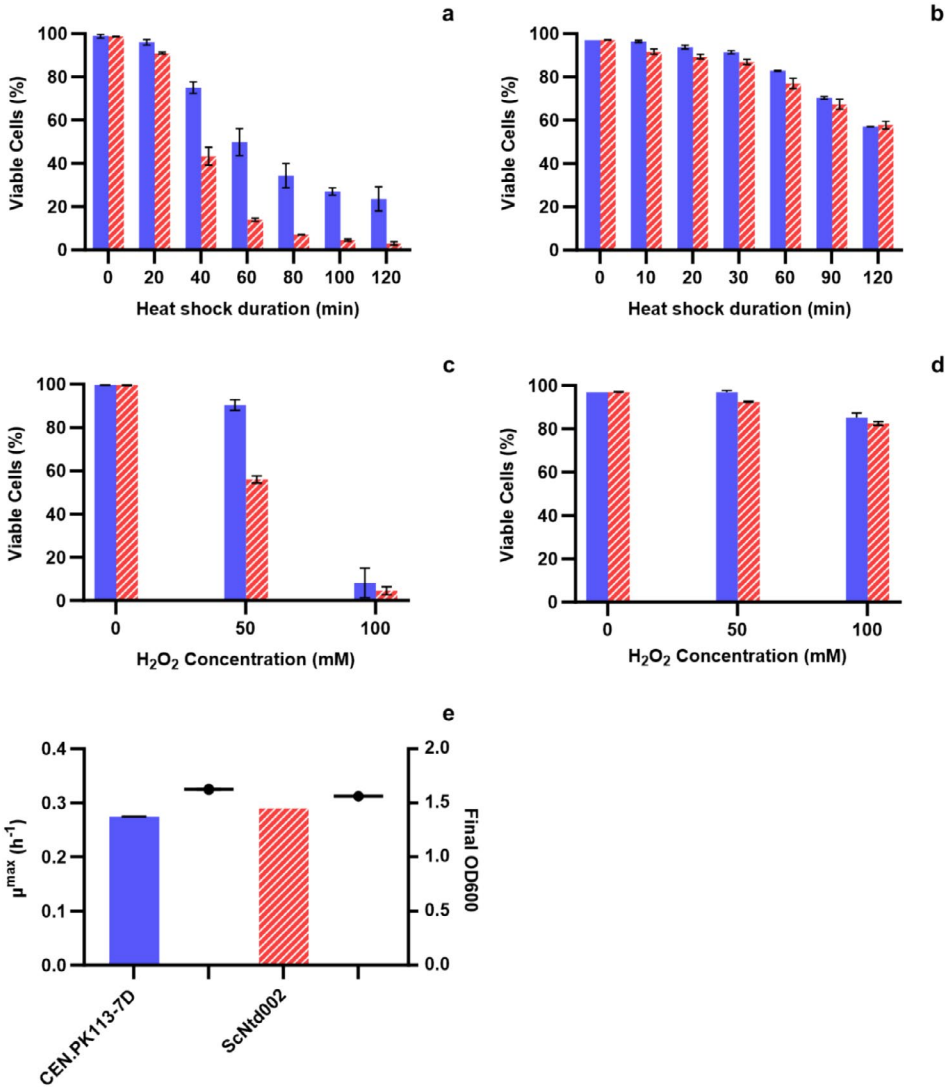

FIGURE 1 | Stress resistance of aerobic shake flask cultures of *S. cerevisiae* CEN.PK113-7D (blue) and ScNtd002 (red). Average values of two biological duplicates are shown, error bars indicate the standard deviation. (a) Viability of exponentially growing cultures exposed to 53°C. (b) Viability of stationary phase cultures exposed to 53°C. (c) Viability of exponentially growing cultures exposed to hydrogen peroxide. (d) Viability of stationary phase cultures exposed to hydrogen peroxide. (e) Specific growth rates (bars, left y-axis) and final OD600 values (dots, right y-axis) in shake flask cultures.

did not significantly impact maximal biomass yields (p-value = 0.19). This is in line with the observed similar final biomass concentrations in batch cultures (Figure 1e). Based on flow cytometry analysis, we did not observe any viability or morphology changes (data not shown). Combined with the similar maximal growth rates, this strongly suggests that Msn2 and Msn4 do not play an essential role in biosynthesis or cell cycle progression at low to high growth rates.

3.3 | Reduced GSR Increases Maintenance Energy Requirement

The maintenance coefficients of the parental yeast strain CEN.PK 113-7D and the Δ*msn2*Δ*msn4* deletion strain ScNtd002 were also estimated using linear regression of q_S versus μ data of chemostat cultures (Figure 2a). In contrast to the maximal biomass yields, the deletion of GSR transcription factors did result in a significantly higher m_S value of the mutant strain (0.082 ± 0.045 mmol/g/h) compared to the parental strain (0.066 ± 0.032 mmol/g/h) (p-value = 0.031, ANCOVA). According to Pirt's maintenance energy concept, maintenance energy is described as cellular functions beyond new biomass formation, at the theoretical zero growth rate (Pirt 1982). Our results highlight an important role of GSR in energy management.

The expression of GSR proteins is inversely correlated with specific growth rate (Regenberg et al. 2006; Castrillo et al. 2007; Vos et al. 2016; Xia et al. 2022), Msn2 and Msn4 are more active at very low growth rates. Combined with the observation that Msn2 and Msn4 impact the MER, the MER should not be constant but actually reduce with decreasing growth rate. The glucose consumption for maintenance estimated using extrapolation of the chemostat measurements for the parental *S. cerevisiae* strain CEN.PK113-7D (0.066 mmol/g/h) is higher than the previously reported 0.039 mmol/g/h determined using glucose-limited,

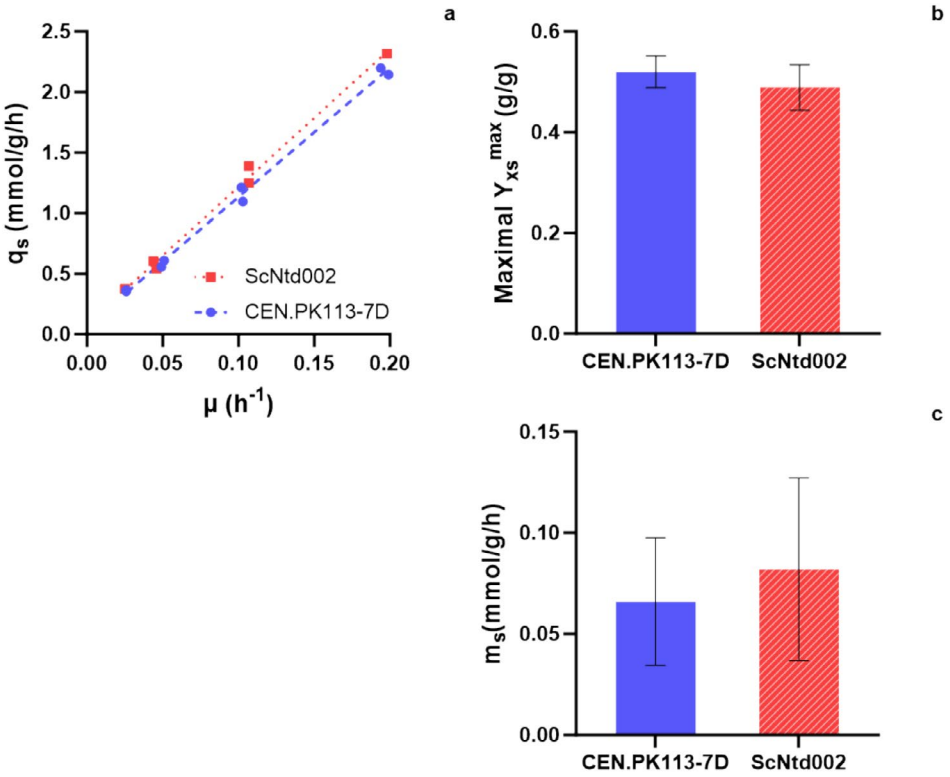

FIGURE 2 | (a) Specific glucose consumption rates versus specific growth rates in aerobic glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D (circles) and ScNtd002 (squares). (b) Maximal biomass yields on glucose (Y_{xs}^{max}) estimated using chemostat cultures of *S. cerevisiae* CEN.PK113-7D (blue) and ScNtd002 (red). (c) Maintenance coefficients estimated using chemostat cultures of *S. cerevisiae* CEN.PK113-7D (blue) and ScNtd002 (red). Shown are estimated values of linear regression for chemostat data, where error bars indicate the uncertainty in the regression.

aerobic retentostat cultivations (Vos et al. 2016). As the media and strain used are the same, this difference might be explained by the growth rates used for the extrapolation; these approach zero (from 0.025 to below 0.001 h-1) in retentostat cultures (Vos et al. 2016).

Chemostat cultures are not well suited to study low dilution rates, as these result in discontinuous, drop-wise feeding and long durations to reach steady state. On the other hand, retentostat cultures are rather labor intensive and time-consuming; near-zero growth rates are typically only reached after 3 weeks of cultivation. Therefore, we opted for an alternative method, fed-batch cultures, to estimate MER. Fed-batch cultures with a constant feed flow rate result in biomass accumulation and a consequent decrease in glucose availability per cell. This reduction in specific glucose uptake rates also results in reduced specific growth rates (Figure 3a). The constant feed rate was chosen such that it resulted in initial growth rates of ~0.1 h^{-1}, which gradually decreased to 0.017 (± 0.001) h^{-1} in our fed-batch cultures. m_S values were estimated by fitting the measurement data to Pirt's model as described in the Materials and Methods, using a maximal theoretical biomass yield of 0.51 g/g based on the chemostat data regression, which was identical to the value found by Verduyn et al. (1991). With this approach, the MER of CEN.PK113-7D was estimated to be 0.040 ± 0.003 mmol/g/h (Figure 3b). This value aligns closely with the previous retentostat estimate by Vos et al. (2016). These findings suggest that indeed MER can be more accurately estimated in fed-batch cultures.

The difference between the MER of the parental and Δ*msn2*Δ*msn4* mutant strains, estimated using fed-batch data, 0.040 ± 0.003 and 0.073 ± 0.007 mmol/g/h respectively, was larger than that between the chemostat-based values (Figure 3c). This observation supports the role of the GSR in maintenance energy saving as the GSR intensifies with decreasing growth rates. Also, under the slower-growing conditions in fed-batch cultures, the difference in maintenance energy coefficients between strains was significant (p-value = 0.01, t-test).

We calculated the observed yield at the end of the batch and the fed-batch phases (Table 2). The difference in observed yield (Y_{XS}) at the end of the fed-batch between the parental (0.43 ± 0.00 g/g) and the mutant strain (0.41 ± 0.01 g/g) is not significant (p-value = 0.20, t-test).

4 | Discussion

In industrial bioprocesses, yeast cells are used to produce food components, pharmaceuticals, biochemicals, or biofuels. Understanding the mechanisms involved in the MER can aid us in optimising these bioprocesses. Any energy saved in maintenance can potentially translate to improved profitability. To understand the role of the GSR in MER, we determined MER of parental and GSR-reduced strains using two different cultivation methods. This showed that the MER is 23% higher in the GSR-reduced strain based on chemostat cultures, while extrapolation from fed-batch culture data revealed an even

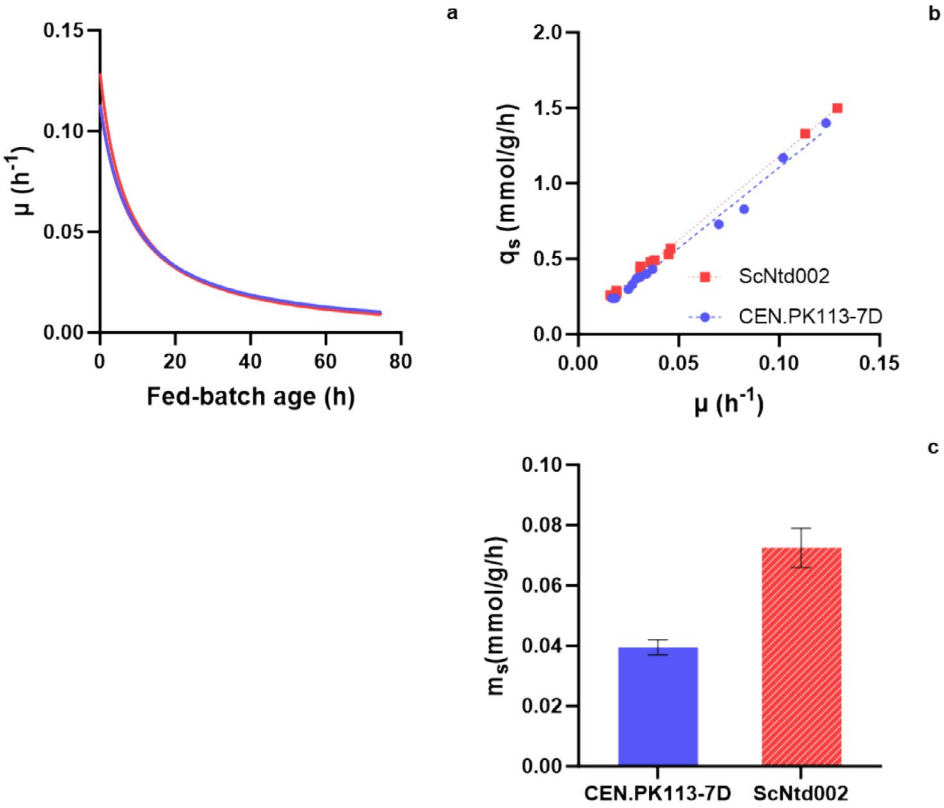

FIGURE 3 | (a) Specific growth rate of fed-batch cultures overtime in average of two replicates. (b) Specific glucose consumption rates versus specific growth rates in aerobic glucose-limited fed-batch cultures of *S. cerevisiae* CEN.PK113-7D (circles) and ScNtd002 (squares). (c) Maintenance coefficients estimated using fed-batch cultures of *S. cerevisiae* CEN.PK113-7D (blue) and ScNtd002 (red). Shown are the average values of duplicate fed-batch cultures, where error bars represent the SEM.

TABLE 2 | Observed biomass yields and maintenance allocation of glucose consumption in aerobic batch cultures and after a 42-h feeding phase.

	Batch phase		End of fed-batch phase	
Strain	**CEN.PK113.7D**	**ScNtd002**	**CEN.PK113.7D**	**ScNtd002**
Y_{XS} (g/g)	0.38 ± 0.03	0.39 ± 0.03	0.43 ± 0.00	0.41 ± 0.01
m_S/q_S (%)			16.5 ± 0.0	28.4 ± 0.6

more pronounced increase of 85%. In other words, when the GSR is reduced, cells invest more energy in cellular maintenance. Thus, the fraction of substrate consumed for maintenance increases (m_S/q_S, Table 2). This results in higher specific glucose uptake rates (q_S) of the mutant strain than of the reference strain at each tested specific growth rate (μ), illustrated by the higher lines for the Δmsn2Δmsn4 strain in Figures 2a and 3b. Higher specific glucose uptake rates correspond to reduced observed biomass yields ($Y_{XS} = \mu/q_S$). At higher growth rates, this effect is less pronounced as a lower fraction of the total glucose consumed is used for maintenance processes. This also masks the effect of higher maintenance requirements on observed final yields in our fed-batch experiments (Table 2). The fed-batch design consisted of a linear feed, which allowed us to test the impact of different growth rates but also meant that most biomass is formed at relatively high growth rates during the preceding batch and initial feed phase. No changes in morphology and viability were observed compared to the parental strain. This indicates that besides the impact on cellular maintenance, Msn2 and Msn4 hardly influenced yeast physiology in the here-studied fed-batch cultures. This is in contrast with the effect that *RIM15* deletion has on extremely calorie-restricted *S. cerevisiae* cells. Deletion of this Greatwall kinase, that among others activates Msn2 and Msn4, resulted in a lower biomass yield, loss of viability and elongated morphologies in anaerobic retentostat cultures (Bisschops et al. 2014). This difference might be explained by the pleiotropic role of Rim15: besides activating the stress response, it also plays a role in cell cycle arrest and reserve carbohydrate accumulation (Bisschops et al. 2014; Moreno-Torres et al. 2015). Compared to the absence of Rim15, deletion of *MSN2* and *MSN4* thus results in a milder phenotype, as only stress response and cellular energetics appear to be affected. The increased maintenance energy requirements especially result in higher catabolic rates, i.e., the conversion of glucose into metabolic energy. This is in line with observations for Sake yeast strains, which are known to lack active Msn2 and/or Msn4 and are characterised by higher ethanol

formation rates (Watanabe et al. 2011). Under anaerobic conditions, ethanol formation directly corresponds to ATP generation. Our results indicate that this increased ATP generation might be, at least in part, due to increased maintenance energy requirements.

The larger difference in estimated MER between the two strains in fed-batch cultures is not due to differences in the estimated MER values for the mutant strain; the lack of transcription factors Msn2 and Msn4 only caused a minor decrease in estimated MER of 11% between chemostat and fed-batch cultures. In contrast, the MER extrapolated for the parental strain under a wide growth rate range in chemostat cultures is 67% higher than the value obtained via slow-growing fed-batch cultures. The value based in fed-batch cultures is furthermore in line with previously reported m_S values in retentostat cultures (Vos et al. 2016). This could indicate that both these cultivation modes, retentostat and fed-batch, allow MER estimation with higher accuracy. An alternative explanation could be that the MER varies with growth rate, decreasing under slower-growing conditions, in part due to the activation of the general stress response (GSR).

Msn2 and Msn4 are the primary activators of the GSR and regulate genes that encode proteins involved in carbohydrate accumulation, protein folding and degradation, DNA repair and intracellular signalling (Gasch et al. 2000). Among these functions, there are several candidate processes that could contribute to energy saving. Protein synthesis is one of the most energy-expensive cellular processes. The expression of molecular chaperones, such as the heat-shock protein Ssa3, can limit the need for protein re-synthesis. Even though these chaperones utilise energy from ATPase to stabilise native proteins, this is more energy efficient than the complete recycling of the same protein (Sharma et al. 2010; Goloubinoff et al. 2018; Fauvet et al. 2021). Furthermore, other Hsp70 family chaperones prevent the aggregation of proteins (Clare and Saibil 2013). Besides the stabilisation of existing proteins, Msn2 and Msn4 impact the levels of proteins that allow easier degradation of unfolded proteins. This plays a role in eliminating protein traffic and toxic accumulation, which may contribute to energy saving (Gupta et al. 2018; Higgins et al. 2020; O' Neill et al. 2020). Misfolded or damaged proteins can be eliminated via the ubiquitination proteasome system (UPS) or autophagy. While Msn2/4 controlled Cdc48 and Hsp104 play a role in UPS (Bodnar and Rapoport 2017; Gupta et al. 2018; Harari et al. 2022; Ji et al. 2022; Li et al. 2024), Msn2/4 controlled ATG proteins play a role in autophagy. It has been shown earlier that autophagy is involved in cell homeostasis and MER (Chen et al. 2023). The elimination of the major kinase Atg1, which activates macroautophagy, resulted in a 60% increase in maintenance energy requirement and a loss in viability. These authors suggested that autophagy plays a dual role in the energetics of maintenance. Autophagy allows yeast cells to efficiently conserve energy from glucose by enhanced maintenance of mitochondrial functionality, and it recycles macromolecules, thereby contributing to energy saving. The expression of superoxide dismutases (Sod1 and Sod2) and catalases (Ctt1 and Cta1) is also controlled in part by Msn2/4. Neutralisation of superoxide radicals and hydrogen peroxide prevents reactive oxygen species (ROS) caused damage in cellular compartments, which would reduce energy expenditure for cellular repair.

Our study revealed that cells that lack Msn2 and Msn4 are more sensitive to heat and hydrogen peroxide stress in the exponential phase, but not during the stationary phase. Lower resistance has previously been observed for exponentially growing cultures (Berry and Gasch 2008). However, it has also been shown that the expression of genes with STRE motifs in their promoters, i.e., under the control of Msn2/Msn4, is strongly upregulated during the diauxic shift (Boy-Marcotte et al. 1998). Based on this, a larger effect of *MSN2* and *MSN4* deletion on stress resistance in the stationary phase, i.e., after the diauxic shift, was expected (Gasch et al. 2000; Virgilio 2012). However, we did not observe significant differences in stress resistance between stationary phase cultures of the parental strain and the Δ*msn2*Δ*msn4* deletion mutant. The regulon of Heat shock factor 1 (Hsf1), another stress-associated transcription factor, overlaps for 57% with that of Msn2 and Msn4. Hsf1 is also regulated by glucose-signalling pathways, including the Snf1 pathway which regulates energy homeostasis upon glucose depletion (Persson et al. 2022). Thus, activation of Hsf1 due to glucose starvation in the stationary phase might dampen the effect of Msn2 and Msn4's absence. This could also explain the upregulation of *HSP12* observed in the Δ*msn2*Δ*msn4* strain after the exponential phase in plate reader experiments (Figure S1). Although we did not see any change in resistance during the stationary phase, we did observe a change in MER. This can be due to specific energy-saving mechanisms controlled by Msn2/4 and not by other regulator proteins like Hsf1 and Yak1. Furthermore, a few differences between shake-flask stationary phase cultures and slow-growing fed-batch cultures may explain the differential regulation of other transcription factors that mask the effect of Msn2 and Msn4 absence in the first. One important difference between stationary phase and slow growth is glucose starvation versus limitation, but also other stresses occurred in uncontrolled shake flask cultures such as the presence of ethanol and low pH. The conserved transcription factor Hsf1 controls the expression of many protein chaperones and could also contribute to a reduction of maintenance energy requirements. Therefore, its effect on stress resistance and energetics in slow-growing cultures would be relevant to investigate, but this protein is essential in *S. cerevisiae* and was therefore not included in the present study (Solís et al. 2016).

Bodegom's concept of maintenance energy encompasses mechanisms like oxidative stress defence, proofreading, macromolecule turnover and osmoregulation (Van Bodegom 2007). Our findings show that the GSR impacts these processes and significantly contributes to reducing the energy needed for maintenance. Besides this positive effect on cellular energetics, there are additional features of GSR that positively affect bioprocesses. For example, activation of the stress response, via Msn4 and Hac1, has been shown to increase recombinant protein secretion in *K. phaffii* (Zahrl et al. 2023). On the other side, the yeast GSR involves approximately 600 genes, including the targets of Msn2/4. Some of the encoded proteins may not contribute to energy saving or enhanced product formation but rather present a waste of valuable substrate. A more targeted approach might then be designed to optimise energy saving further. Such

an approach could follow the example of (Löffler et al. 2016) who identified energy-consuming targets in *E. coli*, and by their elimination reduced MER while increasing intracellular eGFP yield (Ziegler et al. 2021).

5 | Conclusion and Outlook

Two hypotheses have been postulated to explain the activation of GSR in slow-growing microorganisms. The GSR may prepare cells for adverse changes (Bisschops et al. 2014) and/or the GSR may compensate for lower levels of growth-associated proteins to maintain cellular crowding and thereby optimal reaction rates (Bruggeman et al. 2020). Both these hypotheses present advantages of the increased GSR in slow-growing cells, but do not explain the evolutionary driving force behind this response, that is, the direct in situ benefit for the individual cell. Here we present data that support a third explanation, which is that GSR contributes to energy saving under harsh conditions. Energy saving offers a direct selective advantage, as it frees energy for other cellular functions, including growth. Our chemostat and fed-batch data show that GSR indeed reduces the MER of *S. cerevisiae*. As this response is widely conserved (Ercan et al. 2015; Bruggeman et al. 2020), these results might also be valid for other industrially relevant microorganisms.

By employing a novel method based on fed-batch cultures, we obtained more accurate estimations of maintenance energy requirements than those based on the conventional method that involves multiple chemostat cultures. Our estimates were in line with retentostat cultures. These cultures are, however, much more time- consuming (3 weeks versus 4 days) and resource-consuming and complex due to their continuous nature.

We furthermore showed that MER can be altered independently of maximal biomass yields and with minor effects on other important parameters such as maximal growth rate and viability. These findings highlight the importance of GSR in optimising yeast performance and stress resilience, offering valuable insights for biotechnological applications.

Author Contributions

Nuran Temelli: investigation, writing – original draft, writing – review and editing, methodology, conceptualization, formal analysis. **Simon van den Akker:** investigation, methodology. **Ruud A. Weusthuis:** conceptualization, writing – review and editing. **Markus M. M. Bisschops:** conceptualization, formal analysis, supervision, writing – original draft, writing – review and editing, methodology.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw data are available upon request to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.