Evaluating proteome allocation of *Saccharomyces cerevisiae* phenotypes with resource balance analysis

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Abstract

Saccharomyces cerevisiae is an important model organism and a workhorse in biochemical production. Here, we reconstructed a compact and tractable genome-scale resource balance analysis (RBA) model (i.e., *sc*RBA) to analyze metabolic fluxes and proteome allocation in a computationally efficient manner. Resource capacity models such as *sc*RBA provide the quantitative means to identify bottlenecks in biosynthetic pathways due to enzyme and/or ribosome availability limitations. ATP maintenance rate and *in vivo* apparent turnover numbers (k_{app}) were regressed from metabolic flux and protein concentration data to capture observed physiological growth yield and proteome efficiency and allocation, respectively. Estimated parameter values were found to vary with oxygen and nutrient availability. Overall, this work (i) provides condition-specific model parameters to recapitulate phenotypes corresponding to different extracellular environments, (ii) alludes to the enhancing effect of substrate channeling and post-translational activation on *in vivo* enzyme efficiency in glycolysis and electron transport

chain, and (iii) reveals that the Crabtree effect is underpinned by ribosome availability limitations and reserved protein capacity.

Introduction

Saccharomyces cerevisiae is a prototrophic yeast (unicellular fungus organism) that has been domesticated for making bread and wine since ancient times[1]. It occupies a wide spectrum of natural habitats, ranging from soils, insects, grapes, and leaves and trunks of plant species[1]. The organism is well-known for its ethanol production (and tolerance) even under aerobic conditions (i.e., the Crabtree effect)[2]. *S. cerevisiae* have been considered as a "generally recognized as safe" (GRAS) organism and used extensively in biological research as a model eukaryotic organism[3] and in large-scale fermentation[4]. A prime example is its use in producing bioethanol[5] for which global demand was 28.91 billion gallons in 2019 [6]. *S. cerevisiae* has also been extensively re-engineered by metabolic engineering to produce various compounds such as fatty acid derivatives and biofuels[7], building block organic acids[8], biopharmaceutical proteins[9], natural products[10,11], and food additives[12]. For example, industrial-scale production of the antimalarial drug artemisinin's precursors has been achieved using yeast strain with heterologous expression of *Artemisia annua*'s enzymes[13]. These numerous applications and adaptations stem from the organism's robustness in industrial settings (e.g., resistance to growth inhibitors, pH, osmotic, and ethanol stresses)[14] and a strong engineering foundation established by well-characterized genome sequences and annotations[15,16] and availability of synthetic biology tools[17].

These has been a number of genome-wide systems biology studies for *S. cerevisiae* focusing on cellular expression under growth condition perturbations[18], regulation[19,20], metabolism[21], and

genotype-phenotype correlation[22]. The study of *S. cerevisiae* metabolism has received considerable attention. Starting from the first genome-scale metabolic (GSM) model reconstructed in 2003[23], successive models have been developed with improved coverage of genome and metabolic functions[24,25]. Using as inputs only biomass composition, gene annotations, gene to protein to reaction (GPR) associations, and reaction reversibility information, GSM models have been shown to predict metabolic fluxes and theoretical yields reasonably well[21]. They have been used extensively to suggest genetic perturbation strategies for metabolic engineering[24]. Recently, upgrades of GSM models accounting for protein and enzyme availability limitations have been made to improve model prediction by imposing upper limits to metabolic fluxes. Sánchez and coworkers developed the GECKO framework that imposes flux upper bounds derived from protein concentration measurements[26]. Oftadeh and coworkers presented the expression and thermodynamics flux (ETFL) model that account for cellular expression system and reaction thermodynamics[27]. These models display enhanced predictions for batch culture conditions where nutrients are in excess and enzyme production capacity becomes the bottleneck. Beyond metabolism and expression processes, a whole-cell model containing 26 sub-models within has also been developed by Ye and coworkers to capture holistically cellular processes[28].

In this paper, we put forth a computationally tractable to parameterize and simulate resource balance analysis (RBA) model for *S. cerevisiae* referred to as *sc*RBA that focuses on metabolism and enzyme and ribosome production. The goal is to construct a model with a level of detail that experimental data can support parameter estimation. Sets of growth and non-growth associated ATP maintenance parameters specific to growth conditions are regressed from a large collection of *S. cerevisiae* growth phenotype data[29–48] to accurately predict condition-dependent growth yield.

The inferred ATP maintenance rates increase significantly in the presence of oxygen and under carbon and nitrogen limitation in agreement with known yeast physiology[49–56]. *In vivo* enzyme turnover parameters (k_{app}) (indicating enzyme efficiency) were regressed using multiple measured extracellular fluxes and protein concentration datasets[29,30,34,37]. We found that k_{app} values are often very different than catalogued *in vitro* turnover numbers (k_{cat})[57] which are typically used in proteome allocation metabolic models. Notably for 4 out of 10 enzymes in glycolysis, 4 out of 4 in electron transport chain, ATP synthase, and 10 out of 20 in amino acyl-tRNA synthetase pathways estimated k_{app} values were significantly larger (i.e., by up to 189-fold for hexokinase) than tabulated k_{cat} data reflecting higher *in vivo* enzymatic efficiency. While insight into the exact mechanism for this enhancement is not revealed by the parameterization of the RBA model, there is ample literature evidence[58–64] for the presence and function of *in vivo* metabolons and enzyme post-translational activations. Interestingly, inferred k_{app} values were generally lower for (i) alternate carbon substrates, (ii) anaerobic conditions, or (iii) carbon/nitrogen limitations in accordance with lower enzymatic efficiencies implied by the phenotypic data. The parameterized *sc*RBA model for *S. cerevisiae* successfully predicted ethanol overflow under abundant glucose and oxygen conditions. We found that growth was limited by rRNA capacity rather than enzyme availability. Only about 72% of protein capacity is used for growth-coupled glycolysis and biomass production, implying that glucose can be redirected for the production of ethanol and the replenishment of the NAD^+ pool using the reserved proteome capacity. The *sc*RBA model captured the flux-limiting effect of enzyme and/or ribosomes availability (i.e., as low as below 20% of FBA predicted fluxes) for 65% of metabolic reactions under glucose uptake conditions. *sc*RBA based predicted maximal product yields for 28 biochemicals were sometimes significantly reduced compared to FBA-calculated values. For example, whereas the maximal predicted yield for

succinate was only 21% of FBA yield due to the enzymatically inefficient pyruvate-to-succinate pathway in yeasts, the predicted maximal yield for fatty acid derived products (i.e., free fatty acid, fatty alcohol, and triacylglycerol) were as high as 87-88% of FBA yield values. Overall, this work puts forth the *sc*RBA model for *S. cerevisiae* that draws from condition-specific parameters to improve prediction accuracy. Its applicability is demonstrated through case studies of *S. cerevisiae*'s metabolic flux and product yield limit estimations.

Methods

RBA model reconstruction

The model *sc*RBA consists of (macro)molecules and reactions for metabolism and cellular machinery production linked through steady-state mass balance constraints as in FBA[65] (see Fig. 1A for a schematic representation). Here, we provide data sources necessary for reconstruction and briefly explain the model elements linking (macro)molecules and reactions. The reconstruction method is described in detail in the Supplementary Methods, with user instructions, formulation (adapted from Goelzer et al., 2011[66]), and indexing. Metabolites and metabolic reactions are ported from *iSace*1144 (available at https://github.com/maranasgroup/iSace_GSM). Blocked reactions identified by flux variability analysis[67] were excluded from *sc*RBA. Protein translation reactions entail amino acids (in the form of charged-tRNA), cofactors, and energy in the form of GTP and ATP[68,69] with stoichiometric coefficients designed to match the corresponding protein sequences[16] using the Uniprot database[70]. The mass action contribution of ribosomes is set to be proportional to the sum of all protein translation fluxes. Ribosomes, in turn, are synthesized from proteins and rRNAs. In yeast, most of the genes (i.e., 1,201 out of 1,208

genes in the model) are encoded in the nucleus chromosome and translated by the nuclear ribosome. The remaining mitochondrial genes[71] (i.e., 7 genes in the model) are translated using reactions that utilize the mitochondrial ribosome which is treated as separate from the cytosolic counterpart. The stoichiometric coefficients for the proteins and rRNAs associated with ribosome production are obtained from the SGD database[16] and ribosome structure observations[72]. rRNA relative ratios are sourced from the RNAcentral database[73]. Enzymes are formed from the corresponding protein subunits whose stoichiometric coefficients are obtained from the Uniprot database[70]. Biomass precursor producing reactions are included in *sc*RBA to inventory all enzymes, ribosomes, and all other macromolecules needed to form biomass. Precursors (and their relative compositions) of DNA, lipids, carbohydrates, metal ions, sulphate, phosphate, and cofactors are ported from the GSM model *iSace*1144. Based on experimental macromolecular measurements[29,38,74,75] the mass fractions of most macromolecules are assumed to remain invariant except for protein, RNA, and carbohydrate fractions that change with increasing growth rate (see Supplementary Data 1). Instead of reconstructing multiple models with different biomass coefficients at different growth rates, the biomass reaction is recast as a set of precursor sink reactions whose fluxes are equal to the coefficients multiplied by the growth rate. We ensure that the biomass molecular weight is always 1 g $mmol^{-1}$ so that growth yield and rate predictions are consistent[76,77]. Detailed biomass formulation is provided in the Supplementary Methods and Supplementary Data 1.

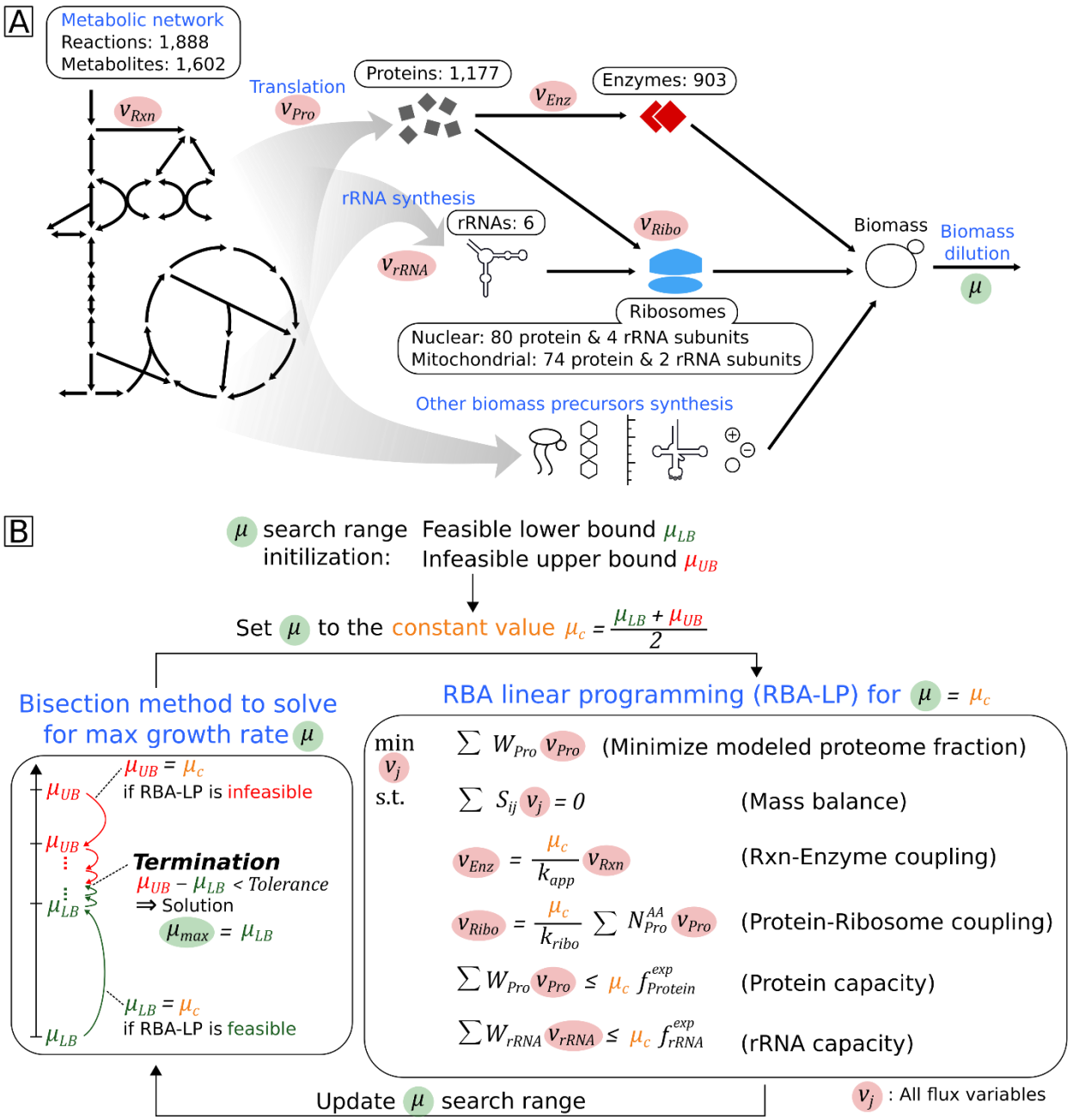

Fig 1 | (A) Schematic representation of the *sc*RBA model (macro)molecular participants and reactions. (B) Overview of bisection method and the RBA linear programming (RBA-LP) formulation that are solved iteratively to obtain the maximal growth rate. Flux variables are highlighted in red and the growth rate variable is highlighted in green. The topology of all *sc*RBA model captured variables are shown in Fig. 1A. Model parameters are briefly explained in the text and formulation details are available in the Supplementary Methods.

The total amount of protein, enzyme, and ribosome produced is determined by the reaction-enzyme and protein-ribosome coupling constraints and limited by the protein and rRNA capacity constraints (see Fig. 1B for a formulation overview, Supplementary Methods for the complete formulation details, and Goelzer et al., 2011 [66] for derivations). The k_{app} parameter values in the reaction-enzyme coupling constraint are derived from experimental flux and proteomics data (see "Estimation of *in vivo* k_{app}" in Methods). In the protein-ribosome coupling constraint, the protein sequence length values (N_{Pro}^{AA}) are taken from the SGD database[16] and the ribosome efficiency parameter (k_{ribo}) is fitted using growth phenotype data[30]. From the experimentally derived[78] average value of 10.5 amino acids elongated per ribosome per second, we re-parameterized the k_{ribo} value by successively increasing it from 10.5 in increments of 0.1 until the predicted growth rate matched the highest reported experimental value of 0.49 h^{-1} (in rich media)[30]. This was met for a slightly higher value of k_{ribo} of 13.2 amino acids per ribosome per second. In the *sc*RBA model, enzyme and ribosome production is limited by the experimentally measured protein and rRNA levels[29,38,74,75] through capacity constraints. Molecular weights of protein and rRNA (i.e., W_{Pro} and W_{rRNA}) are used to convert molar amounts to grams which are set to less than the experimentally found limits (i.e., $f_{Protein}^{exp}$ and f_{rRNA}^{exp}) in the capacity constraints.

*sc*RBA directly accounts for only proteins participating in metabolism and translation/elongation. Proteins involved in other processes such as protein folding chaperone and cellular maintenance are not functionally part of *sc*RBA. We assumed that the modeled proteome including metabolic and ribosomal proteins is 55% of the total proteome [27]. The cost of producing the remaining 45% is accounted for in an aggregate manner assuming average amino acid composition[74]. In resource allocation models this is formulated by adding a reaction producing a non-functional so-called

dummy protein[27,79]. The model accounts explicitly the six rRNA species that are part of ribosomes (see Supplementary Methods for details) which constitute as much as 80% of total RNA[80]. For computational efficiency, mRNA and tRNA demand (i.e., reserving 5% and 15% of total RNA, respectively) are accounted for in an aggregate manner assuming average composition[23]. Similar to the proteome, reaction producing a non-functional RNA is added to the model. *S. cerevisiae* maintains reserved ribosome[78] and enzyme[35] capacity which makes up the difference between experimentally observed and required amounts (estimated under nitrogen limited conditions[35,78]). The reserve proteome capacity is maintained to prepare cells for changes in growth conditions in the same manner that reserve ribosome capacity enables faster growth immediately upon nutrient availability upshift[35,78]. In model, the amounts of non-functional protein and RNA representing reserved capacity are treated as fitted variables so as to recapitulate reserved capacity being present or exhausted depending on growth conditions.

Estimation of ATP maintenance rates

ATP maintenance rates are parameters used in both FBA and RBA model to account for the energy cost of replicating cells. Growth-associated ATP maintenance (GAM) (in mmol gDW^{-1}) rate captures the energy demand per unit of produced biomass. Non-growth associated ATP maintenance (NGAM) (in mmol gDW^{-1} h^{-1}) rate captures the energy demand associated with cellular processes such as repair and maintenance[81]. GAM_{FBA} (i.e., GAM in FBA model) and NGAM parameters were regressed from growth phenotype datasets recorded different growth rates using the FBA model *iSace*1144. For every dataset, ATP maintenance rate was estimated by maximizing flux through the ATP hydrolysis reaction (i.e., $ATP + H_2O \rightarrow ADP + P_i + H^+$)

subject to experimental extracellular fluxes and growth rate[29–37,41–48]. NGAM parameter was equal to the maximal ATP hydrolysis flux estimated from data of growth-arrested conditions[38–40]. GAM_{FBA} is the slope of a linear regression of maximal ATP hydrolysis flux vs. growth rate values whereas the intercept is the NGAM value. GAM_{RBA} (i.e., GAM in RBA model) is estimated by subtracting from GAM_{FBA} value the portion equivalent to protein translation elongation's energy cost, which is approximately 2 mmol of ATP per mmol of amino acid[27]. The subtracted amount is 7.6 mmol ATP gDW^{-1}, derived from experimental amino acid measurements[74]. The NGAM parameter is estimated from growth-arrested conditions data where neither biomass nor protein synthesis is underway and thus the parameter is the same for both FBA and RBA. Different GAM_{FBA} and NGAM parameter sets were regressed from datasets under the following growth conditions: (i) (nutrient-abundant) batch and anaerobic or microaerobic, (ii) C-limited chemostats and anaerobic or microaerobic, (iii) batch or C-limited chemostats and aerobic, (iv) N-limited chemostats and aerobic. Experimental flux inputs and calculated results are recorded in Supplementary Data 2.

Estimation of *in vivo* k_{app}

k_{app} was calculated by dividing estimated intracellular metabolic fluxes by experimental enzyme concentrations[82] (see Supplementary Methods for the workflow and Supplementary Data 3 for details). From literature-reported data[29,30,34,37], different k_{app} parameter sets were determined for growth conditions of: (i) (nutrient-abundant) batch/glucose, (ii) batch/galactose, (iii) batch/maltose, (iv) batch/trehalose, (v) C-limited chemostats/glucose D = 0.1 h^{-1} and (vi) D = 0.3 h^{-1}, and (vii) N-limited chemostats/glucose D = 0.1 h^{-1} and (viii) D = 0.3 h^{-1}.

Growth maximization, flux variability analysis, and predicted yield using *sc*RBA and FBA

An overview of the RBA optimization model that identifies the maximal growth rate is provided in Fig. 1B. By fixing the growth rate the *sc*RBA model is converted into a linear programming LP formulation (i.e., RBA-LP) which can be efficiently solved. An iterative method is employed to converge the upper (infeasible) and lower (feasible) bounds on growth rate within a tolerance criterion of 10^{-5} h^{-1}.

In analogy to flux variability analysis (FVA)[67], lower and upper bounds of reaction fluxes can be calculated using *sc*RBA and FBA models by updating the objective function of the model (see Fig. 1B) to the minimization or maximization of the flux in question and imposing the experimental glucose uptake rate of 13.2 mmol gDW^{-1} h^{-1} and growth rate of 0.42 h^{-1} [30]. Experimental (absolute) glucose uptake and growth rates were used in the simulations to be consistent with model parameters derived from absolute flux and concentration measurements. Flux ranges under FBA and RBA are contrasted to elucidate the role of capacity constraints on the flux allocation flexibility. Maximal compound production rate was identified by maximizing the corresponding (sink) exchange reaction flux variable subject to a glucose uptake of 13.2 mmol gDW^{-1} h^{-1} [30] and growth rate set at a minimum of 0.1 h^{-1}. Hexadecanoic acid and hexadecanol (i.e., C_{16}) were used as proxies in model for *in vivo* mixture of free fatty acids and fatty alcohols of different chain lengths, respectively. Heterologous pathways for the synthesis of tested compounds were reconstructed based on previous studies[7,83–109], as necessary. The RBA-predicted maximal production yield (i.e., $Y_{p,max}^{RBA}$, in g g-Glucose^{-1}) was calculated using the following equation:

$$Y_{p,max}^{RBA} = \frac{MW_p \, v_{p,max}^{RBA}}{MW_{Glc} \, v_{Glc}^{RBA}} \quad \text{(Eq. 1)}$$

where $v_{p,max}^{RBA}$ is the RBA-predicted maximal production rate, MW_p is the product molecular weight, v_{Glc} is the RBA-predicted glucose uptake rate, and MW_{Glc} is the molecular weight of glucose. FBA-calculated maximal production yield (i.e., $Y_{p,max}^{FBA}$) was determined using Eq. 1 but with FBA-predicted rather than RBA-predicted quantities. Fluxes are in mmol gDW^{-1} h^{-1} and molecular weights are in g $mmol^{-1}$.

Software implementation

COBRApy[110] with IBM ILOG CPLEX solver (version 12.10.0.0) were used for FBA model optimization. The FBA model *iSace*1144 is available at https://github.com/maranasgroup/iSace_GSM[111]. General Algebraic Modeling System (GAMS) programming language (version 39.1.0, GAMS Development Corporation) with Soplex solver (version 6.0)[112] was used for RBA model k_{app} parameterization and optimization. Input files as excel spreadsheets were used to build the RBA model in GAMS format. Python 3.6 was used as the central platform to run all mentioned processes. All scripts and input and output files are available in the GitHub repository https://github.com/maranasgroup/scRBA.

Results

Estimation of growth and non-growth associated ATP maintenance

ATP maintenance rate parameters, GAM and NGAM are necessary to accurately account for the fraction of glucose uptake apportioned towards energy production and ultimately growth[77].We estimated GAM and NGAM parameters values using an ATP synthase proton/ATP ratio of 10/3. This reflects the fact that the *S. cerevisiae* ATP synthase consists of 10 c-ring for every 3 F_1F_0 subunits[113] resulting in 10 proton molecules translocated across mitochondrial membrane per 3 ATP molecules produced. Note that earlier GSM models used a proton/ATP ratio of 12/3 [23,114]. An overview of the literature-reported experimental datasets, methods, and GAM/NGAM values is provided in Fig. 2. We calculated from metabolic fluxes of growth-arrested cells[38–40] the NGAM values (in mmol gDW^{-1} h^{-1}) for (i) aerobic C-limited (NGAM = 1.0), (ii) anaerobic C-limited (NGAM = 1.0), and (iii) aerobic N-limited (NGAM = 3.9). Obtained results reveal a largely unchanging NGAM value across C-limited aerobic and anaerobic conditions even though earlier studies pointed at some small differences[39]. New results are likely due to the updated ATP synthase reaction stoichiometry reflecting recent literature information[113]. Note that the calculated NGAM value is nearly 4-fold higher under nitrogen-limited conditions indicating more energy is expended towards cellular maintenance due to the limited nitrogen pool.

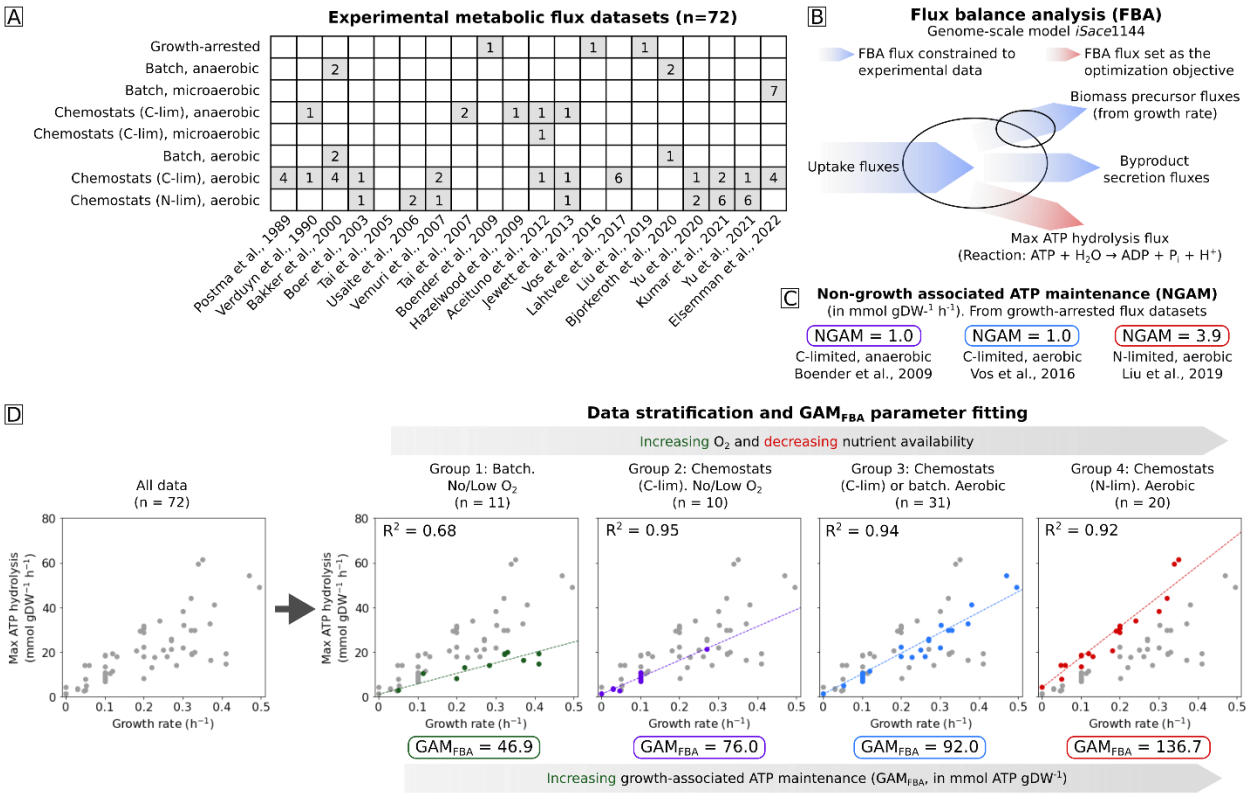

Fig. 2 | Parameterization of growth and non-growth associated ATP maintenance. (A) Summary of the experimental datasets. (B) Overview of the FBA procedure to calculate maximal ATP hydrolysis rate. (C) Non-growth associated ATP maintenance (NGAM) values. (D) Growth-associated ATP maintenance (GAM) values. R^2 is the coefficient of determination for the linear regression to determine the GAM value as the slope. Overall, GAM and NGAM values increase under nutrient limitation and in the presence of oxygen.

GAM_{FBA} values (in mmol gDW^{-1}) are regressed from metabolic flux datasets at different growth rates. Significantly different GAM_{FBA} values were inferred for subsets of data under different experimental oxygen and nutrient availability (see Fig. 2D). This means that GAM_{FBA} values must be tailored to the growth conditions to maintain fidelity of prediction. The lowest GAM value (i.e., GAM_{FBA} = 46.9) is associated with anaerobic batch conditions where nutrients are abundant. Carbon limitation (i.e., GAM_{FBA} = 76.0), presence of oxygen (i.e., GAM_{FBA} = 92.0), or nitrogen limitation (i.e., GAM_{FBA} = 136.7) increase GAM_{FBA} by 1.6-fold, 2-fold, and 2.8-fold, respectively. In the presence of oxygen, energy consumption is diverted towards dissipating reactive oxygen

species from respiration[49,50]. Respiration also requires functional mitochondria whose synthesis and damage repair require energy input[51,52]. The lower GAM_{FBA} value under anaerobic conditions may also be in part due to *S. cerevisiae* adaptation to conditions with abundant fruit-borne sugar but low oxygen availability due to diffusion limitations[115]. Under glucose-limitation, higher ATP maintenance rate is likely needed for the synthesis of the catabolic catalytic apparatus to scavenge alternate carbon sources other than glucose (e.g., through the Snf1 regulatory pathway)[53]. While this is an important adaptation in the natural habitat, it is counterproductive in laboratory settings where alternative carbon sources are not provided. Under N-limitation, higher ATP maintenance rate is associated with the degradation of selected proteins (e.g., ribosomal proteins)[54,55] to replenish depleted pools of amino acids and other N-containing compounds[56]. Because nitrogenous metabolite pools are not conserved by downregulating protein synthesis but rather by engaging an ATP-consuming protein synthesis-degradation cycle, this leads to a significantly higher GAM_{FBA} value under N-limitation. Overall, we estimated condition-specific GAM and NGAM values and provided hypotheses for mechanistic bases for *S. cerevisiae*'s varied ATP maintenance under different growth conditions. Interestingly, we observed that under P-limitation[36] ATP maintenance does not follow a linear trend (as shown in Fig. 2D) and GAM value (i.e., slope) becomes dependent on the degree of limitation (see Supplementary Fig. 1).

Estimation of *in vivo* apparent k_{app} values

Enzyme turnover numbers k_{app} are RBA model parameters that directly affect proteome allocation needs as their product with enzyme levels must match the observed metabolic flux values. Underestimated values for k_{app} results in higher then needed requirements for protein levels and

vice-versa. Even though *in vitro* turnover numbers (k_{cat}) for many reactions are available in the literature and biochemical databases such as BRENDA[57], they are not immediately usable in RBA models. This is because k_{cat} entries do not capture the nuances of the *in vivo* environment (e.g., sub-saturation of enzyme, substrate channeling, post-translational modifications, etc.) that can dramatically alter enzymatic efficiencies[82,116]. In model *sc*RBA, we instead rely on k_{app} values supported by measured metabolic fluxes and corresponding enzyme levels. Assuming that the *in vivo* substrate concentration is below the saturated level, k_{app} values are by definition lower than k_{cat} values[57] (i.e., in the Michaelis-Menten expression, $k_{app} = k_{cat}([S]/(K_M + [S])) \leq k_{cat}$). However, we found in agreement with earlier studies[82,117] that the reverse is true for several enzymes (i.e., 46 out of 132 enzymes in *E. coli* based on available data[82]) indicating that enzyme efficiency is often enhanced *in vivo* through possibly substrate channeling and/or enzyme activation (see Fig. 3 for a visual and Supplementary Data 4 for tabulated values). Enzymes with experimental evidence confirming *in vivo* activity enhancement are reported in Table 1. Enzymes without direct evidence but with predicted marked enhancements *in vivo* are reported in Table 2 as candidates for further testing. Among these candidates is included a hypothetical model of a metabolon involving the ATP synthase proposed for yeast in analogy to the mammalian counterpart[118]. We also found that k_{app} values are lower than k_{cat} for four enzymes in the glycolysis metabolon[58] (see Fig. 3) which suggests that the reducing effect of enzyme sub-saturation is stronger than any enhancing effect of substrate channeling. We find that *in vivo* enhancements occur predominantly in high-flux glycolysis, electron transport chain, and ethanol fermentation alluding to a mechanism to reduce proteome investment for high-capacity enzymes. Note that proteome allocation needs towards these pathways would have been significantly higher if the *in vivo* k_{cat} values were unaltered from the *in vitro* ones. This makes sense as pathways with high flux

would be under a stronger selection to achieve *in vivo* k_{app} enhancements through a variety of mechanisms in comparison to low flux routes. This result further reinforces the need to estimate and utilize condition-specific *in vivo* k_{app} values to faithfully recapitulate *in vivo* enzymatic efficiency and predict proteome allocation with RBA models.

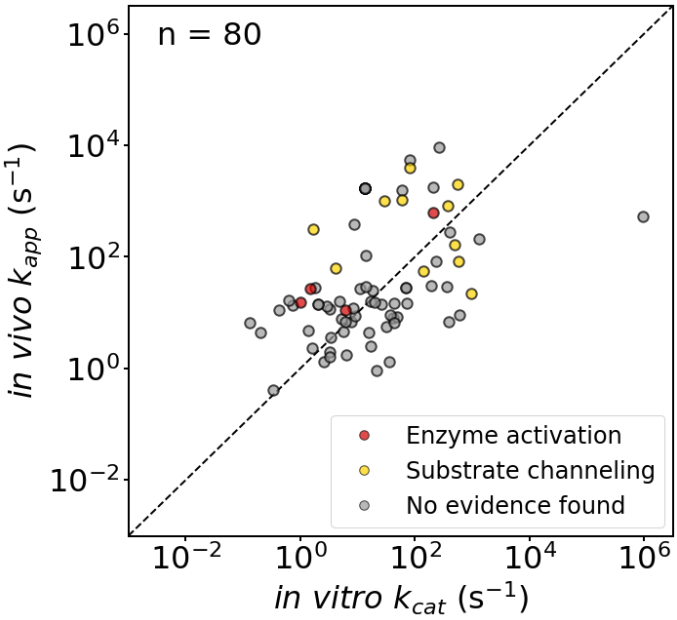

Fig 3 | Comparison of *in vivo* k_{app} vs. *in vitro* k_{cat} values. k_{app} values for the growth condition of batch, aerobic, glucose, and minimal (YNB) media were used in comparison. Experimentally validated enzymes with enhanced *in vivo* efficiency are indicated by red and yellow dots. Gray dots indicate enzymes for which we did not identify such information.

Table 1 | List enzymes whose k_{app} exceed k_{cat} values with independent experimental confirmation

Enzyme	Associated proteins*	k_{app} / k_{cat}	Evidence for *in vivo* activation or channeling
Hexokinase	HXK1, HXK2, GLK1	191	Glycolysis metabolon [a]
Ubiquinol:ferricytochrome-c reductase (complex III)	QCR2, QCR6, QCR7, QCR8, QCR9, QCR10, COB, COR1, RIP1, CYT1	48	Electron transport chain (ETC) metabolon [b]
Glyceraldehyde 3-phosphate dehydrogenase	TDH1, TDH2, TDH3	35	Glycolysis metabolon [a]

Succinate dehydrogenase (ubiquinone) (complex II)	SDH1, SDH2, SDH3, SDH4, SDH9	18	ETC metabolon [b]
Glutamyl-tRNA synthetase	GUS1, ARC1	18	The protein ARC1 is required as a cofactor[62]
Fructose-bisphosphate aldolase	FBA1	15	Glycolysis metabolon [a]
Seryl-tRNA synthetase	SES1, DIA4	15	Enzyme is activated by the protein PEX21[64].
NADH:ubinquinone oxidoreductase	NDI1	3.6	ETC metabolon [b]
Pyruvate decarboxylase	PDC1, PDC5, PDC6	3.0	Activity enhancement *in vivo* by the enzyme SIT4 is observed[63].
Ferrocytochrome-c:O_2 oxidoreductase (complex IV)	COX1, COX2, COX3, COX4, COX5A, COX5B, COX6, COX7, COX8, COX9, COX12, COX13	2.2	ETC metabolon [b]
Methionyl-tRNA synthetase	MES1, ARC1	1.8	The protein ARC1 is required as a cofactor [62]

*Associated proteins are protein subunits or isozymes.

[a] Glycolysis metabolon: Glycolysis enzymes form a metabolon organized along the F-actin filaments[58].

[b] ETC metabolon: ETC's complex III and IV together form the supercomplex III-IV[59]. NDI1 enzyme and ETC's complex II interact with the supercomplex III-IV[60,61].

Table 2 | List of enzymes whose k_{app} exceed k_{cat} values by at least 10-fold without experimental evidence for an enhancement mechanism

Enzyme	Associated proteins*	k_{app} / k_{cat}
Nucleoside diphosphate kinase	YNK1	130
ATP synthase	ATP1, ATP2, ATP3, ATP4, ATP5, ATP6, ATP7, ATP8, ATP14, ATP15, ATP16, ATP17, ATP18, ATP19, ATP20, OLI1, TIM11	68
Isoleucyl-tRNA synthetase	ILS1	52
L-Alanine transaminase	ALT1	44
Inorganic diphosphatase	PPA2	35
Histidyl-tRNA synthetase	HTS1	27
Homocitrate synthase	LYS21	27
Pyruvate carboxylase	PYC1, PYC2	26

Valyl-tRNA synthetase	VAS1	22
Glycyl-tRNA synthetase	GRS1, GRS2	19
Cystathionine gamma-lyase	CYS3	15

*Associated proteins are protein subunits or isozymes.

k_{app} parameter sets were subsequently regressed separately under different growth conditions (see Section 2.3) to assess their impact on enzyme efficiency. Perturbation instances from the reference condition (i.e., batch, aerobic, glucose, minimal (YNB) media) include (i) from aerobic to anaerobic, (ii) from minimal to rich (YNB + amino acids) media, (iii) from batch to chemostats (C- or N-limited), and (iv) from glucose to an alternative carbon substrate. Plots of k_{app}/k_{app}^{ref} ratio values are shown in Fig. 4. k_{app}/k_{app}^{ref} values reflect increased, constant, or reduced enzymatic efficiency under the perturbed from the reference condition. Overall, we found that replacing glucose with an alternate substrate leads to on an average as much as 85% reduction in enzymatic performance (i.e., when switching to trehalose) (see Fig. 4). This enzymatic performance reduction is mirrored in the observed slower growth rates (GR) on galactose (GR = 0.17 h^{-1}), maltose (GR = 0.28 h^{-1}), and trehalose (GR = 0.05 h^{-1}) compared to (GR = 0.42 h^{-1}) for glucose. *sc*RBA results allude to likely strong adaptation of *S. cerevisiae* for proteome-efficient growth on glucose but not for the other three sugar substrates. Specifically, under galactose growth, galactose 1-phosphate accumulates while the fructose 6-phosphate pool is depleted[36] suggesting the formation of a metabolic bottleneck between: (i) UDP-glucose – hexose-1P uridylyltransferase, (ii) UDP-glucose 4-epimerase, or (iii) phosphoglucomutase. Under maltose utilization, the maltose/proton symporter (uptake) and the alpha-glucosidase (maltose to glucose reaction) are likely rate-limiting as *S. cerevisiae* is susceptible to maltose hypersensitivity shock[119]. Under trehalose growth, the turnover number of the first enzymatic step, trehalase (k_{app} of 355 s^{-1}), is

comparable to the one for the glucose growth, hexokinase (k_{app} of 319 s^{-1}). However, a closer look at the measured enzyme concentrations[30,37] reveals an approximately 60-fold lower availability of trehalase (i.e., 0.22 nmol enzyme gDW^{-1}) compared to hexokinase (i.e., 14 nmol enzyme gDW^{-1}) for trehalose and glucose growth, respectively.

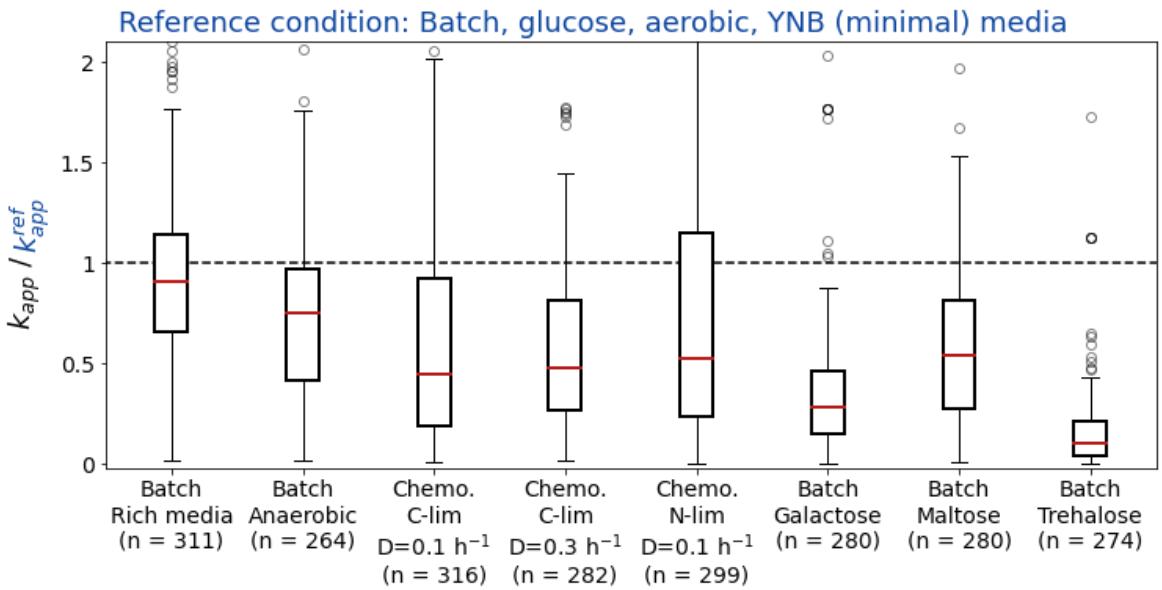

Fig. 4 | Distribution of estimated *in vivo* k_{app} ratios between perturbed and reference conditions. Distributions are visualized using standard box plots (i.e., box: interquartile range (IQR), whiskers: 1.5*IQR, and dots: outliers). The value of "n" indicates the number of overlapping reactions of the respective two sets of reactions whose k_{app} can be estimated from available flux and protein measurements.

Lack of oxygenation and nutrient limitation generally negatively affect enzymatic efficiency (see Fig. 4). Under C- and N- limitation, network-wide efficiency reduction is likely due to depleted intracellular metabolite pools[56] which introduce substrate level limitations for many enzymes. Under anaerobic conditions, two-fold efficiency reduction (i.e., $k_{app}/k_{app}^{ref} \leq 0.5$) are predicted for enzymes in the TCA cycle performing biosynthesis role, ethanol fermentation (i.e., specifically alcohol dehydrogenase), fatty acid biosynthesis and elongations, and nucleotide biosynthesis.

Amino acid supplementation to the (minimal) YNB media does not appear to significantly affect enzyme efficiency (see Fig. 4). Overall, using model *sc*RBA, we found that by estimating condition-specific *in vivo* k_{app} values we can elucidate changes in overall enzymatic efficiency utilization as a function of growth conditions and extracellular nutrient availability.

_sc_RBA model simulation of Crabtree-positive phenotypes

We next contrasted *sc*RBA model predictions against experimental growth phenotypes and proteome allocations at varying glucose uptake rates[29,30,33–37,39,41–44,47]. GAM_{RBA}, NGAM, and *in vivo* k_{app} values for batch aerobic conditions were used in the simulations. Overall, model *sc*RBA recapitulated both the Crabtree-negative phenotype (i.e., no ethanol overflow) at low glucose uptake rates and the Crabtree-positive phenotype (i.e., ethanol overflow) at high glucose uptake rates (see Fig. 5A). The translation parameter k_{ribo} (amino acids per ribosome per second) was set at 13.2 (see Methods) which is only slightly higher than the value of 10.5 estimated from dynamic labeled peptide measurements[78]. Note that FBA using biomass yield as the objective function predicts the Crabtree-negative phenotype but not the Crabtree-positive phenotype as growth rate will simply scale with glucose uptake rate without an upper limit. This implies that in the *sc*RBA model growth rate is limited by rRNA and protein capacity constraints. *sc*RBA predicts that rRNA capacity is exhausted for cells that grow at the maximal rate of 0.47 h^{-1} which matches the experimental derived growth rates (see Fig. 5B). In contrast, to fully utilized rRNA capacity only 72% of protein capacity is needed to produce biomass at the maximal growth rate. This means that more than one fourth of the proteome capacity can be allocated for other cofactor-balanced pathways such as ethanol production and/or complementary glycolysis enzymes.

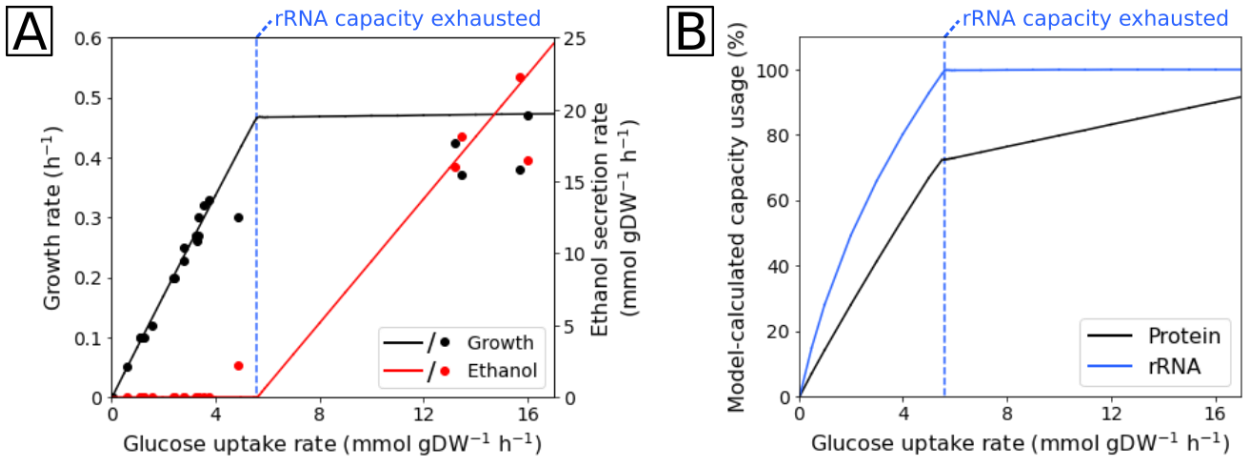

Fig. 5 | Model-simulated vs. experimental growth phenotypes and protein and rRNA capacity usage at varying glucose uptake rates. (A) Predicted (lines) and experimental (dots) growth and ethanol secretion rates at different glucose uptake rates. (B) Protein and rRNA capacity usage at different glucose uptake rates. Overall, model *sc*RBA determines that limited rRNA capacity prevents faster growth and excess protein capacity accommodates ethanol overflow.

Model *sc*RBA identifies that ethanol overflow is a consequence of remaining proteome capacity upon rRNA capacity exhaustion. This raises the question of why rRNA and protein capacities in *S. cerevisiae* are not apportioned to facilitate even faster growth rates. For example, *Escherichia coli* has a much larger RNA capacity (i.e., 20.5% [120] vs. 6.6% gRNA gDW^{-1} in *S. cerevisiae*[74]) and a slightly more efficient ribosome (i.e., k_{ribo} of 17 [121] vs. 13.2 in *S. cerevisiae* (this work)). This enables *E. coli* to access a significantly higher maximal growth rate than *S. cerevisiae* (i.e., max growth of 1.2 h^{-1} [122] vs. 0.49 h^{-1} in *S. cerevisiae*[30]). While at first glance this is counter-intuitive, the slower growing ethanol overflow phenotype offers a number of evolutionary advantages: (i) out-competing other microbes in sugar consumption, (ii) producing ethanol that is toxic to bacterial competitors, and (iii) adapting easily to anaerobic conditions with ready to deploy respiro-fermentative proteome allocation[111,123]. The adaptive advantage of *S. cerevisiae*'s proteome was demonstrated in anaerobic and cyclically oxygenated cultures where higher abundances of *S. cerevisiae* cells competing with the respiratory-favoring yeast *Issatchenkia*

orientalis were measured[111]. Overall, model *sc*RBA results corroborate literature-reported observations and strongly suggest that rRNA limitations coupled with reserved protein capacity are the key drivers of the Crabtree effect in *S. cerevisiae*.

Effect of protein capacity limit on metabolic flux upper bounds and maximal product yields

Enzyme(s) availability bottlenecks can add additional barriers to reaching FBA calculated maximum theoretical limits. Identifying these yield-limiting enzymes is important so as to guide specific gene overexpression strategies remedying these shortcomings without wasting resources on enzymes that are not limiting. To this end, we contrasted the calculated flux bounds (i.e., FVA analysis) using model *sc*RBA (with k_{app} parameters for batch aerobic conditions typically used in compound production) and model *iSace*1144 using FBA. RBA/FBA absolute upper bound flux ratios are calculated for 800 flux-carrying metabolic reactions under glucose uptake conditions (see Fig. 6A for a histogram and Supplementary Data 5 for details). RBA/FBA ratios are less than 20% for as many as 516 out of 800 flux-carrying reactions (see Fig. 6A), indicating that catalytic resource limitations as encoding in model *sc*RBA are propagated to most reactions in the metabolic network. In contrast, two spontaneous reactions, 101 coupled to biomass production reactions, six glucose uptake associated reactions, and fifteen product secretion reactions are (nearly) unconstrained by proteome allocation (i.e., ratio value > 90%). In central metabolism, FBA (through FVA analysis) allows for maximal glycolysis and pentose phosphate pathway (PPP) fluxes that are up to an order of magnitude larger than the glucose uptake rate (i.e., 13.2 mmol gDW^{-1} h^{-1}) (see Fig. 6B and Supplementary Data 5). These very high fluxes are caused by activating ATP-consuming cycles. For example, the FBA-calculated maximal flux of

phosphofructosekinase (ID: PFK_c) reaction in glycolysis is 225 mmol gDW^{-1} h^{-1} which contains an ATP-consuming cycle (i.e., 95% of total flux) with fructose bisphosphate phosphatase reaction in gluconeogenesis. These cycles are retained in FBA because without any additional constraints extra glucose can be used to produce ATP at a yield of up to 25.6 mol ATP / mol glucose. Imposing protein and ribosome availability constraints in RBA greatly reduce the extent of ATP-consuming cycles (see Fig. 6B and 6C). For example, the RBA-calculated PFK_c maximal flux is 27.6 mmol gDW^{-1} h^{-1} which is nearly 10-fold smaller than the FBA-calculated one. The same "tightening" of the upper bound effect through RBA applies to the fluxes of succinate dehydrogenase (ID: SUCDq6_m) and malate dehydrogenase (ID: MDH_m) that are part of the reduced cofactor cycling between cytosol and mitochondria.[124–126] The flux around the cycle is determined exactly by the mitochondrial proton gradient. Therefore, protein capacity constraints are needed to control flux through futile cycles, especially for organisms with highly active overflow metabolism. Enzyme availability also limits TCA cycle fluxes to approximately 80% of the metabolic limit determined by the (glucose-derived) acetyl-CoA surplus (see Fig. 6B and 6C). In contrast, fluxes of the six non-cycling glycolysis reactions are well resolved through FBA as they are fully coupled to the pre-specified glucose uptake (see Fig. 6C). Counterintuitively, their upper bounds are slightly higher using RBA than with FBA by about 1.5 – 1.8% (see Fig. 6C). This is because a slightly higher glycolysis/pentose phosphate pathway (PPP) split ratio for a given glucose uptake is predicted by optimizing NADPH usage in the *sc*RBA model. The lower flux through the NADPH-generating PPP is due to the fact that the actual NADPH needs for amino acid synthesis (accounting in detail by RBA) are slightly less than the lumped amount of the stoichiometric description used in FBA.

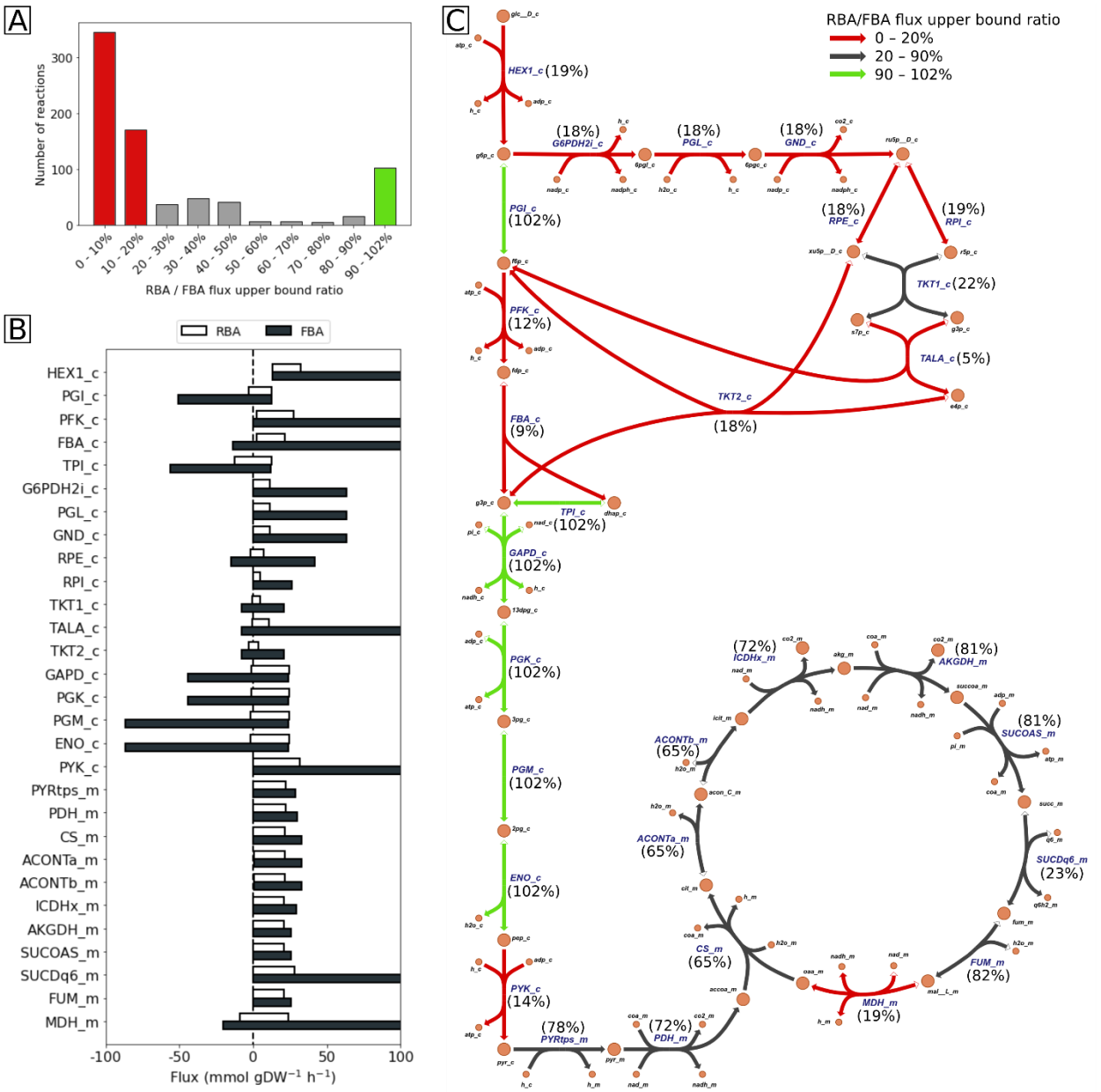

Fig. 6 | *sc*RBA model's metabolic flux variability analysis accounting for the protein capacity limit. (A) Histogram of RBA/FBA flux upper bound ratio values. (B) RBA- (white bars) and FBA-calculated (black bars) flux ranges for reactions in central metabolism subject to experimental glucose uptake and growth rates[30]. Reaction IDs are in BiGG format[127] and reaction details are available in the *sc*RBA github repository. (C) Central metabolism network (drawn by the Escher software[128]) with overlayed reaction IDs and corresponding RBA/FBA flux upper bound ratio values (annotated as colors of arrows).

Next, we evaluated the effect of protein capacity limits on the production yields for 28 compounds by contrasting their maximal yields from *sc*RBA and *iSace*1144 models (see Table 3 and Supplementary Data 5 for RBA-predicted fluxes and proteome allocations). Overall, the maximal yields for 26 out of 28 product metabolites are only marginally restricted by protein capacity with RBA-calculated yields well within 80-100% of the FBA-calculated values (see Table 3). Butanediol, citramalate, ethanol, and lactate productions are least affected with RBA-predicted yields retained at 100% of FBA-calculated yields. This is consistent with the previously determined high protein reserve capacity of *S. cerevisiae* for overflow metabolism. In contrast, succinate and reticuline maximal yields are significantly affected by the protein capacity limit (i.e., down to 21% and 68% of FBA-calculated yields, respectively). Succinate production is directly limited by the enzyme efficiency of its cytosolic pathway. We calculated for *S. cerevisiae* k_{app} values of 13, 30, 71, 1,582 s^{-1} for pyruvate-to-succinate enzymes compared to k_{app} values of 539 and 612 s^{-1} for pyruvate-to-ethanol enzymes. This kinetic bottleneck is absent in *E. coli* whose pyruvate-to-succinate enzyme k_{cat} values (archived in BRENDA[57]) are 250, 540, 931, and 1,150 s^{-1}. Model *sc*RBA-predicted succinate yield (i.e., 0.20 g g-Glucose^{-1}) matches the experimentally observed yield ranges for engineered *S. cerevisiae* strains ranging from 0.11 for an unevolved strain[88] and 0.43 for a laboratory evolved strain[129]. These achieved succinate yields are much lower than the FBA-calculated maximum theoretical yield of 0.95 or alternatively that of an engineered *E. coli* strain (i.e., 0.81)[130]. As demonstrated in this succinate case study RBA results can provide a roadmap for relieving production bottlenecks through gene overexpression and/or replacement of enzymes with more efficient heterologous versions. The production of reticuline, on the other hand, is not limited by the efficiency of the enzymes in the biosynthetic pathway but rather by the proteome cost of recharging the needed cofactors (i.e., two NADPH and three S-adenosyl

methionine (SAM) per reticuline molecule)[98]. Using model *sc*RBA we predicted that supplying NADPH (i.e., through PPP) for reticuline consumes only 1.6% of total available proteome. However, the recovery of SAM (C_1 donor) from S-adenosyl homocysteine (SAH) for reticuline production is approximately 20-fold more proteome consuming than recovering NADPH taking up to 37% of the total proteome. The significant metabolic burden of recovering cofactor SAM has also been reported for *E. coli* where a 12-fold increase in reticuline titer was achieved when the dopamine-to-reticuline conversion occurred *ex vivo* with the growth medium was supplemented in excess with SAM[131]. The costliest product metabolite in terms of NADPH consumption is fatty alcohol requiring 16 moles of NADPH per mole of C_{16}. Despite this high NADPH cost, the corresponding protein allocation for NADPH recharging is only 6.4% of the total proteome. *sc*RBA results demonstrate that a high demand for expensive-to-recharge cofactors can create significant proteome allocation burdens that can negatively affect maximal production yields. This suggests that more enzyme efficient cofactor recharging variants may be a promising route for de-bottlenecking flux through the product biosynthetic pathway. Although protein capacity is not flagged as an issue for most metabolic products, experimentally achieved yields are usually significantly lower than RBA model predicted values except for ethanol and L-lactate (see Table 3). This underperformance may suggest that there is likely untapped potential to further optimize yield through the application of metabolic engineering strategies. Model *sc*RBA predicted proteome allocations for the maximal production of 28 metabolic products are provided in the Supplementary Data 6. These values can help guide further engineering strategies by contrasting with experimentally elucidated quantitative proteomic levels to better guide further strain redesign efforts.

Table 3 | RBA-predicted, FBA-predicted, and literature-reported experimental yields for 28 products

Product	Precursors	$Y_{p,max}^{RBA}$	$Y_{p,max}^{FBA}$	$\frac{Y_{p,max}^{RBA}}{Y_{p,max}^{FBA}}$	Y_{p}^{exp} ##	Pathway and yield reference
Free fatty acid	Fatty acyl-CoA	0.26	0.30	87%	0.1	[83]
Fatty alcohol	Fatty acyl-CoA	0.24	0.27	88%	0.005	[7]
Triacylglycerol ++	Triacylglycerol	0.26	0.29	88%	0.14	[94]
Citramalate	Acetyl-CoA and pyruvate	0.77	0.77	100%	N/A	[103]
n-Butanol	(Acetyl-CoA and pyruvate) or L-threonine	0.32	0.35	91%	0.04	[103]
Polyhydroxybutyrate	Acetoacetyl-CoA	0.52	0.54	97%	0.13	[104]
Artermisinic acid	Farnesyl diphosphate	0.23	0.28	83%	N/A	[105]
Sesquiterpene	Farnesyl diphosphate	0.26	0.31	84%	0.003	[106,107]
Ethanol ++	Ethanol	0.48	0.49	100%	0.44	[108]
L-Lactate	Pyruvate	0.90	0.91	100%	0.69	[109]
(2R,3R)-Butanediol	Pyruvate	0.49	0.49	100%	0.11	[84]
Isobutanol	Isobutanol	0.33	0.39	85%	0.006	[85]
3-Hydroxypropionic acid	Malonyl-CoA	0.69	0.76	91%	0.13	[86]
3-Hydroxypropionic acid	L-Alanine	0.81	0.84	96%	0.14	[87]
Succinate ++	Succinate	0.20	0.93	21%	0.11	[88,129]
Malate ++	Malate	1.16	1.18	98%	0.31	[89]
Shikimate ++	Shikimate	0.70	0.71	99%	N/A	[90]
cis,cis-Muconate	3-Dehydroshikimate	0.60	0.61	99%	0.07	[91]
p-Hydroxybenzoate	Chorismate	0.51	0.52	98%	0.07	[92]
p-Aminobenzoate ++	p-Aminobenzoate	0.42	0.50	85%	0.01	[93]
2-Phenylethanol	L-Phenylalanine	0.35	0.36	99%	0.08	[95]
Styrene	L-Phenylalanine	0.29	0.30	98%	0.001	[96]
p-Coumaric acid	L-Tyrosine	0.48	0.49	98%	0.15	[97]
Reticuline	L-Tyrosine	0.24	0.35	68%	N/A	[98]
(2S)-Naringenin	L-Tyrosine and malonyl-CoA	0.43	0.45	96%	N/A	[99]
Resveratrol	L-Tyrosine and malonyl-CoA	0.36	0.38	96%	0.0002	[100]
Glycerol ++	Glycerol	0.75	0.77	97%	0.5	[101]
1,3-Propanediol	Glycerol	0.49	0.57	86%	N/A	[102]

Optimization constraints: growth rate = 0.1 h^{-1}, glucose uptake rate's upper bound = 13.2 mmol gDW^{-1} h^{-1} (referencing experimental glucose uptake rate [30]). $Y_{p,max}^{FBA}$ and $Y_{p,max}^{RBA}$ (in g g-Glucose^{-1}): FBA- and RBA-calculated maximal theoretical yields. Y_{p}^{exp}: literature-reported experimental yields.

++: These metabolites are produced with native pathways

##: "N/A", or not available, is designated to metabolites with experimental studies where only titers were reported. Citramalate and shikimate were intermediates for other terminal metabolite productions and thus their yields were not reported. A higher yield of 0.43 g-succinate g-glucose^{-1} was reported for a laboratory-evolved strain.

Discussion

Model *sc*RBA provides insights onto *S. cerevisiae* metabolism under a variety of growth conditions by imposing enzyme and ribosome availability limitations on top of mass balances and biomass synthesis needs. *sc*RBA was designed to reduce the number of variables and parameters to only the ones that can be supported by the available proteomic and fluxomic data. Biological processes not contributing to the functional parts of the model (e.g., transcriptional machinery) are modeled in an aggregate manner as sinks for redox and carbon resources without tracking individual steps (see Methods). This makes *sc*RBA more computationally tractable in analyzing metabolic proteome allocation compared to models of larger scope[27,28]. If details on transcription and mRNA availability are available then the ETFL framework[27] provides an alternative. Whole-cell modeling[28] has also been performed for *S. cerevisiae* but computational efficiency and missing parameter values (as noted by the authors) remain a challenge. A distinguishing feature of *sc*RBA is that parameters GAM, NGAM, and k_{app} are derived directly from *in vivo* data thus enabling the condition-specific prediction of growth yield, rate, and proteome allocation. We found significant variation in these parameters depending on nutrient and oxygen availability as well as carbon substrate choice. It is unclear whether parameter values will ultimately converge to the ones for the reference conditions upon adaptation through laboratory evolution for different growth conditions, substrates, and/or genetic backgrounds as seen for *E. coli*[116].

*sc*RBA results suggest that biomass production under maximum growth consumes all available rRNA but only part of the proteome capacity in *S. cerevisiae*. This leaves reserve capacity for ethanol overflow to be carried out providing a mechanism for recharging NAD^+ and an electron sink. The predicted link between reserve proteome capacity and metabolic overflow in *S.*

cerevisiae raises a question on its generality. Analogous overflow phenotypes are present for *E. coli* with acetate[122] and cancer cells with lactate[132] overflow. A quantitative framework similar to *sc*RBA could in principle be applied to confirm the preserve of excess proteome at the point of rRNA exhaustion.

The key design feature of *sc*RBA to use k_{app} as opposed to k_{cat} values unlocks the opportunity to parameterize many more reactions by leveraging availability of quantitative proteomic and fluxomic datasets[29,30,34,37]. *sc*RBA makes use of 336 k_{app} values pegged to *in vivo* protein and flux measurements whereas only 80 k_{cat} values for *S. cerevisiae* under batch aerobic conditions with glucose as substrate can be recovered from BRENDA[57]. Using k_{app} values also obviates the need to "approximate" missing k_{cat} values with measurements from other organisms[133]. Contrasting *in vivo* k_{app} values with *in vitro* k_{cat} values can be an effective method for leveraging multi-omics data to systematically identify enzymes with *in vivo* enhancements. While the mechanism of enhancements may remain elusive, their presence and extent could reveal important biological insight as to the spatial organization of pathways and regulation of enzymes.

Consistent with metabolomics data[36,56] we observe a lowering in k_{app} values under substrate and/or nitrogen limitations caused by reduced metabolite concentrations. This further reinforces the need to re-estimate k_{app} parameters whenever simulation conditions change. An alternative approach could be to track enzyme turnover and enzyme saturation as carried out in the growth balance analysis (GBA) framework[134]. GBA uses formal (nonlinear) kinetic descriptions (e.g., Michaelis-Menten) for the reaction fluxes and mass balance equations for all macromolecules as in RBA. This enhanced level of detail in description comes at the expense of having to generate absolute intracellular metabolite concentration measurements (on top of flux and enzyme

measurements) as inputs to estimate enzyme saturation parameters (e.g., K_M in the Michaelis-Menten equation). Therefore, even though RBA sacrifices the ability to directly model enzyme saturation, it can quantify proteome allocation at a genome-scale in a computationally and data efficient manner.

Model *sc*RBA is shown to be more apt at recapitulating *S. cerevisiae* metabolic phenotypes compared to the corresponding FBA model *iSace*1144 [111]. In particular, *sc*RBA can both identify reaction step bottlenecks and often pinpoint the functional reason for them such as inefficient enzyme with low k_{app} or proteome costly cofactor cycling. We found that a significant fraction of *S. cerevisiae* reactions has an upper bound that is severely restricted due to proteome allocation limits (i.e., more than 60% have an upper bound that is < 20% of FBA theoretical). Despite this *sc*RBA-predicted product yield calculations are generally only marginally lower than the FBA-based theoretical limit. This is primarily due to the fact that most upper bound restriction by RBA happens for reactions that can participate in ATP-consuming cycles. While this ATP overhead is tolerated in FBA calculations by draining from a large glucose surplus, the associated increased proteome allotments are severely curtailed in RBA calculations. Because product synthesis pathways do not generally involve ATP consuming cyclic steps, reductions in predicted maximum yields under RBA are not overly taxing. It is important to stress that the RBA modeling framework does not account for many other factors that could affect production such as regulatory feedback and metabolite pool bottlenecks[135,136]. For example, release of inhibition of several upstream enzymatic steps by high concentrations of tyrosine and phenylalanine need to be addressed when producing shikimate-derived metabolites in *S. cerevisiae*[90]. Overall, we envision *sc*RBA model as part of a combined experimental and computational toolkit to investigate cellular metabolism and

assess metabolic proteome allocation of *S. cerevisiae*. The relative compact nature of the RBA framework presented herein makes it tractable for non-model organisms leveraging advances in GSM reconstructions[137].

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Data availability

Data is available in the GitHub repository https://github.com/maranasgroup/scRBA.

Code availability

Software scripts are available in the GitHub repository https://github.com/maranasgroup/scRBA. The user manual is provided in the Supplementary Methods.

Author contributions

Conceptualization: H.V.D. and C.D.M; methodology, software, and data curation: H.V.D.; analysis: H.V.D. and C.D.M.; resources, supervision, and funding acquisition: C.D.M.; writing: H.V.D. and C.D.M.

Competing interests

The authors declare no competing interests for this manuscript.

References

1. Parapouli, M., Vasileiadis, A., Afendra, A. S. & Hatziloukas, E. *Saccharomyces cerevisiae* and its industrial applications. *AIMS Microbiol.* **6**, 1 (2020).
2. Verduyn, C., Zomerdijk, T. P. L., van Dijken, J. P. & Scheffers, W. A. Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. *Appl. Microbiol. Biotechnol. 1984 193* **19**, 181–185 (1984).
3. Nielsen, J. Yeast systems biology: Model organism and cell factory. *Biotechnol. J.* **14**, 1800421 (2019).
4. Hong, K. K. & Nielsen, J. Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. *Cell. Mol. Life Sci.* **69**, 2671–2690 (2012).
5. Majidian, P., Tabatabaei, M., Zeinolabedini, M., Naghshbandi, M. P. & Chisti, Y. Metabolic engineering of microorganisms for biofuel production. *Renew. Sustain. Energy Rev.* **82**, 3863–3885 (2018).
6. U.S. Energy Information Administration (EIA). Biofuels explained - ethanol and biomass-based diesel. (2021). Available at: https://www.eia.gov/energyexplained/biofuels/data-and-statistics.php. (Accessed: 21st January 2022)
7. Hu, Y., Zhu, Z., Nielsen, J. & Siewers, V. Engineering *Saccharomyces cerevisiae* cells for production of fatty acid-derived biofuels and chemicals. *Open Biol.* **9**, (2019).
8. Sandström, A. G. *et al. Saccharomyces cerevisiae*: A potential host for carboxylic acid production from lignocellulosic feedstock? *Appl. Microbiol. Biotechnol.* **98**, 7299–7318 (2014).
9. Nielsen, J. Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. *Bioengineered* **4**, 207–211 (2013).
10. Wang, G., Kell, D. B. & Borodina, I. Harnessing the yeast *Saccharomyces cerevisiae* for the production of fungal secondary metabolites. *Essays Biochem.* **65**, 277–291 (2021).
11. Chen, R., Yang, S., Zhang, L. & Zhou, Y. J. Advanced Strategies for Production of Natural Products in Yeast. *iScience* **23**, 100879 (2020).
12. Seo, S.-O. & Jin, Y.-S. Next-Generation Genetic and Fermentation Technologies for Safe

and Sustainable Production of Food Ingredients: Colors and Flavorings. *Annu. Rev. Food Sci. Technol.* **13**, 18–19 (2022).
13. Kung, S. H., Lund, S., Murarka, A., McPhee, D. & Paddon, C. J. Approaches and recent developments for the commercial production of semi-synthetic artemisinin. *Front. Plant Sci.* **9**, 87 (2018).
14. Peltier, E., Friedrich, A., Schacherer, J. & Marullo, P. Quantitative trait nucleotides impacting the technological performances of industrial saccharomyces cerevisiaestrains. *Front. Genet.* **10**, 683 (2019).
15. Engel, S. R. *et al.* The Reference Genome Sequence of Saccharomyces cerevisiae: Then and Now. *G3 Genes, Genomes, Genet.* **4**, 389–398 (2014).
16. Cherry, J. M. *et al.* Saccharomyces Genome Database: the genomics resource of budding yeast. *Nucleic Acids Res.* **40**, D700–D705 (2012).
17. Liu, Z., Zhang, Y. & Nielsen, J. Synthetic Biology of Yeast. *Biochemistry* **58**, 1511–1520 (2019).
18. Taymaz-Nikerel, H., Cankorur-Cetinkaya, A. & Kirdar, B. Genome-wide transcriptional response of Saccharomyces cerevisiae to stress-induced perturbations. *Front. Bioeng. Biotechnol.* **4**, 17 (2016).
19. Garcia-Albornoz, M. *et al.* A proteome-integrated, carbon source dependent genetic regulatory network in Saccharomyces cerevisiae. *Mol. Omi.* **16**, 59–72 (2020).
20. Gonçalves, E. *et al.* Systematic Analysis of Transcriptional and Post-transcriptional Regulation of Metabolism in Yeast. *PLOS Comput. Biol.* **13**, e1005297 (2017).
21. Nielsen, J. Systems Biology of Metabolism. *https://doi.org/10.1146/annurev-biochem-061516-044757* **86**, 245–275 (2017).
22. Kang, K. *et al.* Linking genetic, metabolic, and phenotypic diversity among *Saccharomyces cerevisiae* strains using multi-omics associations. *Gigascience* **8**, (2019).
23. Förster, J. *et al.* Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. *Genome Res* **13**, 244–253 (2003).
24. Lopes, H. & Rocha, I. Genome-scale modeling of yeast: chronology, applications and critical perspectives. *FEMS Yeast Res* **17**, (2017).
25. Lu, H. *et al.* A consensus S. cerevisiae metabolic model Yeast8 and its ecosystem for comprehensively probing cellular metabolism. *Nat. Commun.* **10**, 1–13 (2019).
26. Sánchez, B. J. *et al.* Improving the phenotype predictions of a yeast genome-scale metabolic model by incorporating enzymatic constraints. *Mol. Syst. Biol.* **13**, 935 (2017).
27. Oftadeh, O. *et al.* A genome-scale metabolic model of Saccharomyces cerevisiae that integrates expression constraints and reaction thermodynamics. *Nat. Commun. 2021 121* **12**, 1–10 (2021).
28. Ye, C. *et al.* Comprehensive understanding of Saccharomyces cerevisiae phenotypes with whole-cell model WM_S288C. *Biotechnol. Bioeng.* **117**, 1562–1574 (2020).
29. Yu, R., Vorontsov, E., Sihlbom, C. & Nielsen, J. Quantifying absolute gene expression profiles reveals distinct regulation of central carbon metabolism genes in yeast. *Elife* **10**, (2021).
30. Björkeroth, J. *et al.* Proteome reallocation from amino acid biosynthesis to ribosomes enables yeast to grow faster in rich media. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 21804–21812 (2020).
31. Hazelwood, L. A. *et al.* Identity of the growth-limiting nutrient strongly affects storage

carbohydrate accumulation in anaerobic chemostat cultures of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **75**, 6876–6885 (2009).
32. Aceituno, F. F. *et al.* Oxygen response of the wine yeast Saccharomyces cerevisiae EC1118 grown under carbon-sufficient, nitrogen-limited enological conditions. *Appl. Environ. Microbiol.* **78**, 8340–8352 (2012).
33. Jewett, M. C. *et al.* Mapping condition-dependent regulation of lipid metabolism in saccharomyces cerevisiae. *G3 Genes, Genomes, Genet.* **3**, 1979–1995 (2013).
34. Lahtvee, P. J. *et al.* Absolute Quantification of Protein and mRNA Abundances Demonstrate Variability in Gene-Specific Translation Efficiency in Yeast. *Cell Syst.* **4**, 495-504.e5 (2017).
35. Yu, R. *et al.* Nitrogen limitation reveals large reserves in metabolic and translational capacities of yeast. *Nat. Commun. 2020 111* **11**, 1–12 (2020).
36. Kumar, K., Venkatraman, V. & Bruheim, P. Adaptation of central metabolite pools to variations in growth rate and cultivation conditions in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* **20**, (2021).
37. Elsemman, I. E. *et al.* Whole-cell modeling in yeast predicts compartment-specific proteome constraints that drive metabolic strategies. *Nat. Commun. 2022 131* **13**, 1–12 (2022).
38. Liu, Y., el Masoudi, A., Pronk, J. T. & van Gulik, W. M. Quantitative Physiology of Non-Energy-Limited Retentostat Cultures of Saccharomyces cerevisiae at Near-Zero Specific Growth Rates. *Appl. Environ. Microbiol.* **85**, (2019).
39. Vos, T. *et al.* Maintenance-energy requirements and robustness of Saccharomyces cerevisiae at aerobic near-zero specific growth rates. *Microb. Cell Fact.* **15**, (2016).
40. Boender, L. G. M., De Hulster, E. A. F., Van Maris, A. J. A., Daran-Lapujade, P. A. S. & Pronk, J. T. Quantitative physiology of Saccharomyces cerevisiae at near-zero specific growth rates. *Appl. Environ. Microbiol.* **75**, 5607–5614 (2009).
41. Postma, E., Verduyn, C., Scheffers, W. A. & Van Dijken, J. P. Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **55**, 468–477 (1989).
42. Verduyn, C., Postma, E., Scheffers, W. A. & Van Dijken, J. P. Physiology of Saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. *J. Gen. Microbiol.* **136**, 395–403 (1990).
43. Bakker, B. M. *et al.* The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in Saccharomyces cerevisiae. *J. Bacteriol.* **182**, 4730–4737 (2000).
44. Boer, V. M., De Winde, J. H., Pronk, J. T. & Piper, M. D. W. The genome-wide transcriptional responses of Saccharomyces cerevisiae grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J. Biol. Chem.* **278**, 3265–3274 (2003).
45. Tai, S. L. *et al.* Two-dimensional transcriptome analysis in chemostat cultures. Combinatorial effects of oxygen availability and macronutrient limitation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 437–447 (2005).
46. Usaite, R., Patil, K. R., Grotkjær, T., Nielsen, J. & Regenberg, B. Global transcriptional and physiological responses of Saccharomyces cerevisiae to ammonium, L-alanine, or L-glutamine limitation. *Appl. Environ. Microbiol.* **72**, 6194–6203 (2006).
47. Vemuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L. & Nielsen, J. Increasing NADH

oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2402–2407 (2007).
48. Tai, S. L. *et al.* Control of the glycolytic flux in Saccharomyces cerevisiae grown at low temperature: a multi-level analysis in anaerobic chemostat cultures. *J. Biol. Chem.* **282**, 10243–10251 (2007).
49. Trancíková, A., Weisová, P., Kiššová, I., Zeman, I. & Kolarov, J. Production of reactive oxygen species and loss of viability in yeast mitochondrial mutants: protective effect of Bcl-xL. *FEMS Yeast Res.* **5**, 149–156 (2004).
50. Farrugia, G. & Balzan, R. Oxidative stress and programmed cell death in yeast. *Front. Oncol.* **2**, (2012).
51. Pfanner, N. & Meijer, M. Mitochondrial biogenesis: The Tom and Tim machine. *Curr. Biol.* **7**, R100–R103 (1997).
52. Krämer, L., Groh, C. & Herrmann, J. M. The proteasome: friend and foe of mitochondrial biogenesis. *FEBS Lett.* **595**, 1223–1238 (2021).
53. Conrad, M. *et al.* Nutrient sensing and signaling in the yeast Saccharomyces cerevisiae. *FEMS Microbiol. Rev.* **38**, 254–299 (2014).
54. Kolkman, A. *et al.* Proteome analysis of yeast response to various nutrient limitations. *Mol. Syst. Biol.* **2**, (2006).
55. Helbig, A. O. *et al.* The diversity of protein turnover and abundance under nitrogen-limited steady-state conditions in Saccharomyces cerevisiae. *Mol. Biosyst.* **7**, 3316–3326 (2011).
56. Boer, V. M., Crutchfield, C. A., Bradley, P. H., Botstein, D. & Rabinowitz, J. D. Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. *Mol. Biol. Cell* **21**, 198–211 (2010).
57. Jeske, L., Placzek, S., Schomburg, I., Chang, A. & Schomburg, D. BRENDA in 2019: a European ELIXIR core data resource. *Nucleic Acids Res.* **47**, (2019).
58. Araiza-Olivera, D. *et al.* A glycolytic metabolon in Saccharomyces cerevisiae is stabilized by F-actin. *FEBS J.* **280**, 3887–3905 (2013).
59. Schägger, H. & Pfeiffer, K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J.* **19**, 1777–1783 (2000).
60. Linden, A. *et al.* A Cross-linking Mass Spectrometry Approach Defines Protein Interactions in Yeast Mitochondria. *Mol. Cell. Proteomics* **19**, 1161–1178 (2020).
61. Boumans, H., Grivell, L. A. & Berden, J. A. The respiratory chain in yeast behaves as a single functional unit. *J. Biol. Chem.* **273**, 4872–4877 (1998).
62. Simader, H. *et al.* Structural basis of yeast aminoacyl-tRNA synthetase complex formation revealed by crystal structures of two binary sub-complexes. *Nucleic Acids Res.* **34**, 3968–3979 (2006).
63. de Assis, L. J., Zingali, R. B., Masuda, C. A., Rodrigues, S. P. & Montero-Lomelí, M. Pyruvate decarboxylase activity is regulated by the Ser/Thr protein phosphatase Sit4p in the yeast Saccharomyces cerevisiae. *FEMS Yeast Res.* **13**, 518–528 (2013).
64. Rocak, S., Landeka, I. & Weygand-Durasevic, I. Identifying Pex21p as a protein that specifically interacts with yeast seryl-tRNA synthetase. *FEMS Microbiol. Lett.* **214**, 101–106 (2002).
65. Orth, J. D., Thiele, I. & Palsson, B. Ø. What is flux balance analysis? *Nat. Biotechnol.* **28**, 245–248 (2010).
66. Goelzer, A., Fromion, V. & Scorletti, G. Cell design in bacteria as a convex optimization

problem. *Automatica* **47**, 1210–1218 (2011).
67. Mahadevan, R. & Schilling, C. H. H. The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. *Metab. Eng.* **5**, 264–276 (2003).
68. Dever, T. E., Kinzy, T. G. & Pavitt, G. D. Mechanism and regulation of protein synthesis in Saccharomyces cerevisiae. *Genetics* **203**, 65–107 (2016).
69. Jackson, R. J., Hellen, C. U. T. & Pestova, T. V. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews Molecular Cell Biology* **11**, 113–127 (2010).
70. Bateman, A. *et al.* UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res.* **49**, D480–D489 (2021).
71. Foury, F., Roganti, T., Lecrenier, N. & Purnelle, B. The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae. *FEBS Lett.* **440**, 325–331 (1998).
72. Desai, N., Brown, A., Amunts, A. & Ramakrishnan, V. The structure of the yeast mitochondrial ribosome. *Science* **355**, 528–531 (2017).
73. Sweeney, B. A. *et al.* RNAcentral 2021: secondary structure integration, improved sequence search and new member databases. *Nucleic Acids Res.* **49**, D212–D220 (2021).
74. Lange, H. C. & Heijnen, J. J. Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **75**, 334–44 (2001).
75. Van Hoek, P., Van Dijken, J. P. & Pronk, J. T. Effect of specific growth rate on fermentative capacity of baker's yeast. *Appl. Environ. Microbiol.* **64**, 4226–4233 (1998).
76. Chan, S. H. J., Cai, J., Wang, L., Simons-Senftle, M. N. & Maranas, C. D. Standardizing biomass reactions and ensuring complete mass balance in genome-scale metabolic models. *Bioinformatics* **33**, 3603–3609 (2017).
77. Dinh, H. V., Sarkar, D. & Maranas, C. D. Quantifying the propagation of parametric uncertainty on flux balance analysis. *Metab. Eng.* **69**, 26–39 (2022).
78. Waldron, C., Jund, R. & Lacroute, F. Evidence for a high proportion of inactive ribosomes in slow-growing yeast cells. *Biochem. J.* **168**, 409–415 (1977).
79. Lloyd, C. J. *et al.* COBRAme: A computational framework for genome-scale models of metabolism and gene expression. *PLOS Comput. Biol.* **14**, e1006302 (2018).
80. Warner, J. R. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **24**, 437–440 (1999).
81. Thiele, I. & Palsson, B. A protocol for generating a high quality genome-scale metabolic reconstruction. *Nat. Protoc.* **5**, 93–121 (2010).
82. Davidi, D. *et al.* Global characterization of in vivo enzyme catalytic rates and their correspondence to in vitro kcat measurements. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 3401–3406 (2016).
83. Yu, T. *et al.* Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. *Cell* **174**, 1549-1558.e14 (2018).
84. Ng, C. Y., Jung, M. Y., Lee, J. & Oh, M. K. Production of 2,3-butanediol in *Saccharomyces cerevisiae* by *in silico* aided metabolic engineering. *Microb. Cell Fact.* **11**, (2012).
85. Wess, J., Brinek, M. & Boles, E. Improving isobutanol production with the yeast Saccharomyces cerevisiae by successively blocking competing metabolic pathways as well as ethanol and glycerol formation. *Biotechnol. Biofuels* **12**, (2019).
86. Kildegaard, K. R. *et al.* Engineering and systems-level analysis of *Saccharomyces cerevisiae* for production of 3-hydroxypropionic acid via malonyl-CoA reductase-

dependent pathway. *Microb. Cell Fact.* **15**, 1–13 (2016).
87. Borodina, I. *et al.* Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via β-alanine. *Metab. Eng.* **27**, 57–64 (2015).
88. Raab, A. M., Gebhardt, G., Bolotina, N., Weuster-Botz, D. & Lang, C. Metabolic engineering of Saccharomyces cerevisiae for the biotechnological production of succinic acid. *Metab. Eng.* **12**, 518–525 (2010).
89. Zelle, R. M. *et al.* Malic acid production by Saccharomyces cerevisiae: Engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. *Appl. Environ. Microbiol.* **74**, 2766–2777 (2008).
90. Averesch, N. J. H. & Krömer, J. O. Metabolic engineering of the shikimate pathway for production of aromatics and derived compounds-Present and future strain construction strategies. *Front. Bioeng. Biotechnol.* **6**, 32 (2018).
91. Wang, G. *et al.* Improvement of cis, cis-Muconic Acid Production in *Saccharomyces cerevisiae* through Biosensor-Aided Genome Engineering. *ACS Synth. Biol.* **9**, 634–646 (2020).
92. Averesch, N. J. H., Prima, A. & Krömer, J. O. Enhanced production of para-hydroxybenzoic acid by genetically engineered Saccharomyces cerevisiae. *Bioprocess Biosyst. Eng.* **40**, 1283–1289 (2017).
93. Averesch, N. J. H., Winter, G. & Krömer, J. O. Production of para-aminobenzoic acid from different carbon-sources in engineered *Saccharomyces cerevisiae*. *Microb. Cell Fact.* **15**, 1–16 (2016).
94. Arhar, S. *et al.* Engineering of Saccharomyces cerevisiae for the accumulation of high amounts of triacylglycerol. *Microb. Cell Fact.* **20**, 1–15 (2021).
95. Hassing, E. J., de Groot, P. A., Marquenie, V. R., Pronk, J. T. & Daran, J. M. G. Connecting central carbon and aromatic amino acid metabolisms to improve de novo 2-phenylethanol production in Saccharomyces cerevisiae. *Metab. Eng.* **56**, 165–180 (2019).
96. McKenna, R., Thompson, B., Pugh, S. & Nielsen, D. R. Rational and combinatorial approaches to engineering styrene production by Saccharomyces cerevisiae. *Microb. Cell Fact.* **13**, (2014).
97. Liu, Q. *et al.* Rewiring carbon metabolism in yeast for high level production of aromatic chemicals. *Nat. Commun. 2019 101* **10**, 1–13 (2019).
98. Deloache, W. C. *et al.* An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. *Nat. Chem. Biol.* **11**, 465–471 (2015).
99. Gao, S. *et al.* Efficient Biosynthesis of (2 S)-Naringenin from p-Coumaric Acid in Saccharomyces cerevisiae. *J. Agric. Food Chem.* **68**, 1015–1021 (2020).
100. Li, M. *et al.* De novo production of resveratrol from glucose or ethanol by engineered Saccharomyces cerevisiae. *Metab. Eng.* **32**, 1–11 (2015).
101. Overkamp, K. M. *et al.* Metabolic engineering of glycerol production in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **68**, 2814–2821 (2002).
102. Rao, Z. *et al.* Engineered *Saccharomyces cerevisiae* that produces 1,3-propanediol from D-glucose. *J. Appl. Microbiol.* **105**, 1768–1776 (2008).
103. Shi, S. *et al.* Metabolic engineering of a synergistic pathway for n-butanol production in Saccharomyces cerevisiae. *Sci. Reports 2016 61* **6**, 1–10 (2016).
104. Kocharin, K., Chen, Y., Siewers, V. & Nielsen, J. Engineering of acetyl-CoA metabolism

for the improved production of polyhydroxybutyrate in Saccharomyces cerevisiae. *AMB Express* **2**, 1–11 (2012).
105. Ro, D. K. *et al.* Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**, 940–943 (2006).
106. Liu, C. L. *et al.* Metabolic engineering strategies for sesquiterpene production in microorganism. *Crit. Rev. Biotechnol.* **42**, (2022).
107. Scalcinati, G. *et al.* Combined metabolic engineering of precursor and co-factor supply to increase α-santalene production by Saccharomyces cerevisiae. *Microb. Cell Fact.* **11**, 1–16 (2012).
108. Ruchala, J., Kurylenko, O. O., Dmytruk, K. V. & Sibirny, A. A. Construction of advanced producers of first- and second-generation ethanol in Saccharomyces cerevisiae and selected species of non-conventional yeasts (Scheffersomyces stipitis, Ogataea polymorpha). *J. Ind. Microbiol. Biotechnol.* **47**, 109–132 (2020).
109. Novy, V., Brunner, B., Müller, G. & Nidetzky, B. Toward "homolactic" fermentation of glucose and xylose by engineered Saccharomyces cerevisiae harboring a kinetically efficient l-lactate dehydrogenase within pdc1-pdc5 deletion background. *Biotechnol. Bioeng.* **114**, 163–171 (2017).
110. Ebrahim, A., Lerman, J. A., Palsson, B. O. & Hyduke, D. R. COBRApy: COnstraints-Based Reconstruction and Analysis for Python. *BMC Syst. Biol.* **7**, 74 (2013).
111. Shen, Y. *et al.* Proteome capacity constraints favor respiratory ATP generation. *bioRxiv* 2022.08.10.503479 (2022). doi:10.1101/2022.08.10.503479
112. Gleixner, A., Steffy, D. & Wolter, K. *Iterative Refinement for Linear Programming*. (2015).
113. Symersky, J. *et al.* Structure of the c10 ring of the yeast mitochondrial ATP synthase in the open conformation. *Nat. Struct. Mol. Biol. 2012 195* **19**, 485–491 (2012).
114. Aung, H. W., Henry, S. A. & Walker, L. P. Revising the representation of fatty acid, glycerolipid, and glycerophospholipid metabolism in the consensus model of yeast metabolism. *Ind. Biotechnol.* **9**, 215–228 (2013).
115. Goddard, M. R. & Greig, D. *Saccharomyces cerevisiae*: a nomadic yeast with no niche? *FEMS Yeast Res.* **15**, 1–6 (2015).
116. Heckmann, D. *et al.* Kinetic profiling of metabolic specialists demonstrates stability and consistency of in vivo enzyme turnover numbers. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 23182–23190 (2020).
117. Chen, Y. & Nielsen, J. In vitro turnover numbers do not reflect in vivo activities of yeast enzymes. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 2108391118 (2021).
118. Clémençon, B. Yeast mitochondrial interactosome model: metabolon membrane proteins complex involved in the channeling of ADP/ATP. *Int. J. Mol. Sci.* **13**, 1858–1885 (2012).
119. Jansen, M. L. A., Daran-Lapujade, P., De Winde, J. H., Piper, M. D. W. & Pronk, J. T. Prolonged maltose-limited cultivation of Saccharomyces cerevisiae selects for cells with improved maltose affinity and hypersensitivity. *Appl. Environ. Microbiol.* **70**, 1956–1963 (2004).
120. Neidhardt, F. C. & Curtiss, R. Escherichia coli *and* Salmonella*: cellular and molecular biology*. (ASM Press, 1996).
121. Young, R. & Bremer, H. Polypeptide-chain-elongation rate in *Escherichia coli* B/r as a function of growth rate. *Biochem. J.* **160**, 185–194 (1976).
122. Basan, M. *et al.* Overflow metabolism in Escherichia coli results from efficient proteome

allocation. *Nature* **528**, 99–104 (2015).
123. Dashko, S., Zhou, N., Compagno, C. & Piškur, J. Why, when, and how did yeast evolve alcoholic fermentation? *FEMS Yeast Res.* **14**, 826–832 (2014).
124. Palmieri, F. *et al.* Identification of mitochondrial carriers in Saccharomyces cerevisiae by transport assay of reconstituted recombinant proteins. *Biochimica et Biophysica Acta - Bioenergetics* **1757**, 1249–1262 (2006).
125. Cavero, S. *et al.* Identification and metabolic role of the mitochondrial aspartate-glutamate transporter in Saccharomyces cerevisiae. *Mol. Microbiol.* **50**, 1257–1269 (2003).
126. Larsson, C. *et al.* The Importance of the Glycerol 3-Phosphate Shuttle During Aerobic Growth of Saccharomyces cerevisiae. *Yeast* **14**, 347–357 (1998).
127. King, Z. A. *et al.* BiGG Models: A platform for integrating, standardizing and sharing genome-scale models. *Nucleic Acids Res.* **44**, (2016).
128. King, Z. A. *et al.* Escher: A Web Application for Building, Sharing, and Embedding Data-Rich Visualizations of Biological Pathways. *PLOS Comput. Biol.* **11**, e1004321 (2015).
129. Otero, J. M. *et al.* Industrial Systems Biology of *Saccharomyces cerevisiae* Enables Novel Succinic Acid Cell Factory. *PLoS One* **8**, e54144 (2013).
130. Zhang, W. *et al.* Metabolic Engineering of Escherichia coli for High Yield Production of Succinic Acid Driven by Methanol. *ACS Synth. Biol.* **7**, 2803–2811 (2018).
131. Matsumura, E. *et al.* Laboratory-scale production of (S)-reticuline, an important intermediate of benzylisoquinoline alkaloids, using a bacterial-based method. *Biosci. Biotechnol. Biochem.* **81**, 396–402 (2017).
132. Liberti, M. V. & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **41**, 211–218 (2016).
133. Bekiaris, P. S. & Klamt, S. Automatic construction of metabolic models with enzyme constraints. *BMC Bioinformatics* **21**, (2020).
134. Dourado, H. & Lercher, M. J. An analytical theory of balanced cellular growth. *Nat. Commun. 2020 111* **11**, 1–14 (2020).
135. Davy, A. M., Kildegaard, H. F. & Andersen, M. R. Cell Factory Engineering. *Cell Syst.* **4**, 262–275 (2017).
136. Foster, C. J., Wang, L., Dinh, H. V., Suthers, P. F. & Maranas, C. D. Building kinetic models for metabolic engineering. *Current Opinion in Biotechnology* **67**, 35–41 (2021).
137. Chen, Y., Li, F. & Nielsen, J. Genome-scale modeling of yeast metabolism: retrospectives and perspectives. *FEMS Yeast Res.* **22**, 1–9 (2022).