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## Metabolic Engineering of Yeast

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Shuobo Shi,<sup>1</sup> Yu Chen,<sup>2</sup> and Jens Nielsen<sup>1,3,4</sup>
<sup>1</sup>State Key Laboratory of Green Biomanufacturing, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, China

<sup>2</sup>Key Laboratory of Quantitative Synthetic Biology, Shenzhen Institute of Synthetic Biology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China

<sup>3</sup>BioInnovation Institute, Copenhagen, Denmark

<sup>4</sup>Department of Life Sciences, Chalmers University of Technology, Gothenburg, Sweden; email: nielsenj@chalmers.se


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## Keywords

 metabolic engineering, yeast cell factory, synthetic biology, computational design, *Saccharomyces cerevisiae*, biorefinery

## Abstract

Microbial cell factories have been developed to produce various compounds in a sustainable and economically viable manner. The yeast *Saccharomyces cerevisiae* has been used as a platform cell factory in industrial biotechnology with numerous advantages, including ease of operation, rapid growth, and tolerance for various industrial stressors. Advances in synthetic biology and metabolic models have accelerated the design–build–test–learn cycle in metabolic engineering, significantly facilitating the development of yeast strains with complex phenotypes, including the redirection of metabolic fluxes to desired products, the expansion of the spectrum of usable substrates, and the improvement of the physiological properties of strain. Strains with enhanced titer, rate, and yield are now competing with traditional petroleum-based industrial approaches. This review highlights recent advances and perspectives in the metabolic engineering of yeasts for the production of a variety of compounds, including fuels, chemicals, proteins, and peptides, as well as advancements in synthetic biology tools and mathematical modeling.

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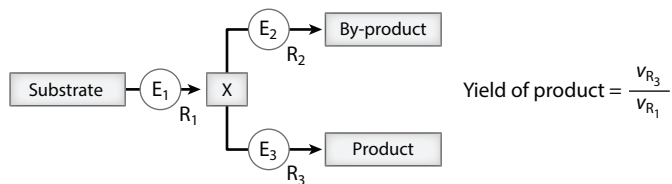
## INTRODUCTION

For more than 6,000 years the yeast *Saccharomyces cerevisiae* has been domesticated for the production of bread, beer, and wine. These processes rely on the efficiency of yeast to rapidly convert sugars into ethanol while tolerating high concentrations of ethanol. Because of this capability, yeast was exploited for large-scale production of ethanol, which can be used as a transportation fuel. In fact, in 1908 Henry Ford initially developed internal combustion engines to rely on ethanol as the key fuel, and only later in the 1920s did the shift to gasoline occur. During the oil crisis of the 1970s, interest in the use of ethanol as a transportation fuel was renewed, and large industrial scale production based on sugar cane and corn starch was established in Brazil and the United States, respectively. Today, yeast-based production of ethanol, as well as its traditional applications, is by far the largest industrial scale application of microbial fermentation processes.

Due to the large industrial application of yeast, *S. cerevisiae* has been extensively studied, and therefore it also became an important model organism (70). The combination of our extensive knowledge of, for example, yeast metabolism and genetic regulation and the capability of yeast to tolerate harsh industrial conditions has made it well-suited as a cell factory for producing a wide range of chemicals (1, 70). Through engineering yeast metabolism, it is possible to redirect flux from ethanol to other products of interest and even to insert synthetic biosynthetic pathways where genes from other organisms are expressed in yeast. This concept is referred to as metabolic engineering (71, 72).

It is generally possible to engineer yeast metabolism through metabolic engineering to produce small amounts of the product of interest, but it is more challenging to produce the product at levels that make it industrially relevant. For this, it is important to improve the titer, rate, and yield (TRY), as recently discussed by Konzock & Nielsen (43).

Optimizing yield is important for maximizing the utilization of the feedstock, generally the carbon source. In ethanol production, more than 50% of the total production costs are associated with the costs of the feedstock, but in this process it is also possible to obtain a high yield; i.e., many processes operate at 90–95% of the maximum theoretical yield of 0.51 g ethanol/g glucose. For the production of other chemicals, it is more challenging to obtain high yields, but if the product's



**Figure 1**

Yields and rates in metabolic networks. Metabolic networks are large and consist of thousands of reactions, but overall yields and rates are determined by how fluxes distribute around the many different branch points within the metabolic network. This is conceptually illustrated for a single branch point. Yield is determined by the relative flux between the flux upstream of the branch point metabolite and the flux leading toward the product of interest. Rate ( $R_1$ ,  $R_2$ ,  $R_3$ ) is determined by the flux toward the product of interest, and improving rate therefore often also results in improved yield. Key determinants for flux control around the branch point are the concentration of the enzymes ( $E_1$ ,  $E_2$ ,  $E_3$ ) and the concentration of the branch point metabolite ( $c_X$ ).

price is higher than that of ethanol, it is also acceptable with a lower yield. Improving yield is directly related to ensuring that flux is directed toward the product of interest, and this means that there should be a focus on so-called branch points within the metabolic network (**Figure 1**). Often metabolic flux can be redirected through the deletion or attenuation of enzyme activities in branches of the metabolism that do not lead to the product of interest (80), but flux can also be redirected by modifying the enzymes working at the branch points (87).

Optimizing rate is important for ensuring efficient utilization of the production facility. If the rate is low, then a large production facility is needed to produce a certain amount of product, and this affects the unit cost of the product. Rate improvement is typically attained by enhancing enzyme performance (45) (i.e., the catalytic efficiency), but the rate can also be improved by increasing the enzyme concentration through overexpressing the gene encoding the enzyme.

Obtaining a high titer of the product is important for ensuring cost-efficient product purification, generally termed downstream processing. If yeast has been engineered to enable high yield and high rate, it is generally possible to obtain a high titer by designing the proper fermentation process. However, in some cases the product is toxic to yeast and therefore it may be necessary to engineer the yeast to improve its tolerance for the product (76).

Here, we review the application of yeast for the production of a variety of chemicals. We also discuss how different mathematical modeling concepts can be applied for the identification of metabolic engineering targets, and we present different methods for engineering yeast. We conclude with several future perspectives on the application of yeast.

## ADVANCES IN THE CONSTRUCTION OF YEAST CELL FACTORIES

Amid growing interest in a biobased economy, yeast cell factories have emerged as a platform for sustainable production of a broad spectrum of products (**Table 1**). These products range from low-value, high-volume commodities such as fuels and commodity chemicals to high-value, low-volume specialties such as specialty chemicals and proteins. The development and optimization of these cell factories for industrial applications typically necessitate enhancements in TRY.

### Fuels

To date, bioethanol represents the most widely consumed liquid fuel synthesized via yeast. Previous efforts have focused on improving bioethanol production from corn starch or molasses feedstocks, resulting in high yields and high titers in industrial production (55, 103). Current research has shifted its focus to the production of ethanol from sugars derived from

**Table 1** Examples of engineered yeast for production of a wide range of different products mentioned in this review

Product	Product application	Carbon source	Production level	Reference
<b>Fuels</b>				
Ethanol	Drop in biofuel	Molasses	114.71 g/L	103
		Corn cob	94.76 g/L	8
Butanol	Drop in biofuel and precursor for jet fuels	Glucose	1.67 g/L	51
		Glucose	0.24 g/L	90
Free fatty acids	Biofuel and platform chemical	Glucose	33.4 g/L	105
		Glucose	0.56 g/L	109
Fatty acid ethyl esters	Biodiesel	Glucose	5 g/L	44
Fatty alcohols	Biofuel and lubricants	Glucose	1.5 g/L	112
Farnesene	Biodiesel and jet fuel	Glucose	28.3 g/L	100
Limonene	Biodiesel and jet fuel	Glucose	2.63 g/L	42
Isoprene	Precursor for jet fuels	Glucose	3.7 g/L	98
<b>Chemicals (including commodity chemicals, fine chemicals, and specialty chemicals)</b>				
3-Hydroxypropionic acid	Commodity chemical	Glucose	71.06 g/L	108
		Glucose and bicarbonate	11.25 g/L	80
Muconic acid	Commodity chemical	Glucose	2.1 g/L	91
Succinic acid	Precursor of various commercial chemicals	Glycerol	45.5 g/L	83
L-phenylacetylcarbinol	An important drug intermediate	Glucose	2.48 g/L	33
7-Dehydrocholesterol	Vitamin D3 precursor	Glucose and glycerol	1.33 g/L	81
Rubusoside	Sugar substitutes	Glucose	1.37 g/L	104
Astaxanthin	Antioxidant and coloring dyes	Glucose	0.446 g/L	48
Carotenoid	Antioxidant and coloring dyes	Glucose	37.39 mg/L	37
Naringenin	Antioxidative and anti-inflammatory effects	Glucose	3.42 g/L	47
Bikaverin	Antibiotic, antifungal, and anticancer properties	Galactose	0.20 g/L	110
Cannabinoid	Psychoactive drugs	Galactose	2.3 mg/L tetrahydrocannabinolic acid; 4.2 µg/L cannabidiolic acid	58
Tropane alkaloid	Anticholinergic drugs	Glycerol	480 µg/L hyoscyamine; 172 µg/L scopolamine	92
Heme	Cofactor of essential enzymes	Glucose	53.5 mg/L	34
Taxa-4(20),11-dien-5α-ol	Precursors of taxol (an anticancer drug)	Galactose	43.65 mg/L	60
Caffeic acid	Pharmaceutical	Glucose	5.5 g/L	7
Ferulic acid	Pharmaceutical	Glucose	3.8 g/L	7
Sclareol	Fragrance molecules	Glucose	11.4 g/L	5
Ginsenoside Rh2	Anticancer drug	Glucose	300 mg/L	114

(Continued)

Table 1 (Continued)

Product	Product application	Carbon source	Production level	Reference
<b>Proteins and peptides</b>				
Insulin and its analogs	Treatment of diabetes	Glucose	Approximately 90 mg/L insulin precursor	53
		Glucose	84 mg/L insulin precursor	28
$\alpha$ -Amylase	Hydrolyzing starch	Glucose	2.5 g/L	30
Hemoglobin	Oxygen carrier and a meat-like flavor	Glucose	18% of total soluble proteins	35
GAPDH-derived peptides	Antimicrobials	Mixture of sugars (sucrose, glucose, and fructose)	A higher inhibitory effect against <i>Brettanomyces bruxellensis</i> (72-fold in synthetic must fermentation and tenfold in carob syrup fermentation)	3
Bacteriocins	Antimicrobials	Glucose	18.4 mg/L plantaricin 423; 20.9 mg/L mundticin ST4SA	85

Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

lignocellulosic biomass, necessitating efficient sugar release in lignocellulosic hydrolysate (61) and the development of highly efficient xylose-utilizing yeast strains (8). For instance, novel active xylose isomerases were identified to construct an efficient xylose-utilizing strain of *S. cerevisiae* (8). Following adaptive laboratory evolution (ALE), this xylose-utilizing strain was evolved to produce ethanol at a titer of 94.76 g/L from pretreated corn cobs. Although ethanol was the initial model for biofuel commercialization, it presents several technical challenges, such as low energy content, miscibility with water, and incompatibility with existing fuel distribution and storage infrastructure. In contrast, other nonethanol biofuels, such as higher alcohols (with more than two carbons), fatty acid derivatives, and terpenes, are more promising alternatives due to their closer chemical resemblance to current petroleum-based fuels.

Biobutanol is a suitable fuel molecule because it has a higher energy content, lower water solubility, and reduced vapor pressure compared with ethanol. Two primary strategies have been used to produce butanol in yeast: heterologous expression of the *Clostridium* pathway and endogenous amino acid degradation pathways. Optimization of the substrate supply, redox balance, and stress tolerance through the *Clostridium* pathway enhanced butanol production in engineered yeasts, achieving a titer of 1.67 g/L (51). Conversely, endogenous butanol production, which relies on amino acid degradation, has shown lower productivity (90). However, the butanol TRY remains lower than that of ethanol, presenting significant challenges that need addressing, and improving butanol tolerance is also required for increased production (16).

Fatty acids and their derivatives, including oils, fatty acid ethyl esters, free fatty acids, and fatty alcohols, can be used as fuels or fuel precursors. Specifically, yeast metabolism has been reprogrammed toward lipogenesis through a comprehensive biological redesign that encompasses metabolic rewiring, directed evolution, and bioprocess optimization (105). Additionally, accumulated fatty acids can be transformed into various derivatives, such as fatty acid ethyl esters (44) and fatty alcohols (112). Identifying efficient enzymes for the production of fatty acid derivatives remains a challenge, which could potentially be addressed through high-throughput screening or protein engineering techniques.

Several terpene-based biofuels, such as farnesene and limonene, can be synthesized by expressing various terpene synthases. Recent research has demonstrated the highly efficient production of farnesene, achieving a yield of 28.3 g/L through combined enzyme and metabolic engineering strategies (100). Similarly, the development of a synthetic pathway, augmented by dynamic

regulation and compartmental engineering, enabled the biosynthesis of limonene at 2.63 g/L (42). These compounds, characterized by their ring structures and methyl branches, exhibit superior fuel performance and can serve as alternatives to diesel and jet fuel. Furthermore, synthetic biology techniques allow for the customization of terpene structures, enhancing their suitability as fuels (113).

## Chemicals

Yeast cell factories have been developed to produce a variety of commodity chemicals. One such chemical, 3-hydroxypropionic acid (3-HP), holds significant industrial applications. By leveraging mitochondrial capabilities, cofactor engineering, and flux optimization at the acetyl-CoA node, researchers achieved a notable 3-HP titer of 71.06 g/L in a meticulously engineered yeast (108). Further advancements enhanced the carbon yield of 3-HP to 0.5625 g 3-HP/g glucose by minimizing native CO<sub>2</sub> emissions and reducing carbon waste (80).

Fine chemicals function as intermediates or active ingredients in pharmaceuticals, agrochemicals, and specialty chemicals. Dicarboxylic acids, such as malate and succinate, are extensively utilized in the fine chemical industry. Noteworthy research has facilitated the production of succinate at a concentration of 45.5 g/L by optimizing the flux between the reductive and oxidative branches of the TCA cycle, as well as by employing mitochondrial transporters (83). Additionally, an inorganic–biological hybrid system has been developed to enable the production of succinate that is more carbon and energy efficient (25).

Expressing secondary metabolite biosynthetic pathways in yeast enables the production of high-value specialty chemicals with diverse applications, including dyes, pigments, agriculture, medicine, nutrition, fragrances, and flavors. Increasing attention has been paid to terpenoids, flavonoids, and polyketides. For instance, astaxanthin, a highly valued antioxidant and coloring terpenoid, was produced in yeast at a concentration of 446.4 mg/L through the integration of spatial regulation, lipid engineering, and dynamic regulation (48). Naringenin, a pharmacologically significant flavonoid, was effectively synthesized using a multipathway synergistic and enzyme engineering strategy, achieving a final titer of 3.4 g/L (47). Additionally, the biosynthesis pathway for the tetracyclic polyketide bikaverin was constructed in yeast, leading to a production level of 202.75 mg/L after alleviating pathway bottlenecks (110).

Beyond the efficient and sustainable production of known compounds, exploring novel synthetic compounds remains another primary objective in synthetic biology. For example, unnatural cannabinoid analogs with customized C3 side chains were synthesized by exploiting enzyme promiscuity (58). Furthermore, computational tools like ATLASx (67) have been utilized to expand the natural biochemical space, resulting in the production of two different classes of tropane alkaloid derivatives: nortropane alkaloids and tropane *N*-oxides (92). Additionally, combinatorial biosynthesis has been used to create novel sesquiterpenoids with unique hydrocarbon scaffolds by combining various sesquiterpene cyclases and P450 oxygenases (93).

## Proteins and Peptides

The production of biopharmaceutical proteins represents one of the fastest-growing sectors within the multibillion-dollar pharmaceutical industry. Yeasts have been utilized to produce approximately 29 biopharmaceuticals currently on the market, accounting for approximately one-sixth of all pharmaceuticals used in human medicine (96). These biopharmaceuticals include primarily vaccines and small proteins for treating conditions such as diabetes and obesity. For example, Novo Nordisk has been producing insulin, one of the earliest and most well-known

biopharmaceuticals, using yeast since 1987; yeast-based production now accounts for half of the global insulin supply (41). Research has shown that multiple factors, including the leader sequence (53) and vesicle trafficking (28), influence insulin production. Additionally, modifications to the insulin sequence have led to the development of insulin analogs designed to optimize pharmacokinetic and pharmacodynamic properties (41). An example is insulin detemir, engineered to extend the duration of insulin's activity (40). Currently, over 70% of biopharmaceuticals are glycoprotein products, and strategies to engineer glycosylation modification processes in yeast have been employed.

Yeasts can produce various industrial enzymes for commercial purposes. These enzymes find applications across various sectors. For instance, enzymes such as amylase, invertase, lipase, and pectinase are crucial for purifying food products and enhancing food components. Numerous strategies have been used to enhance yeast's protein production capabilities. Through combinatorial genetic manipulation, optimally engineered yeast strains have achieved production levels of  $\alpha$ -amylase of 2.5 g/L (30). Furthermore, recent advancements have introduced high-throughput (99) and rational design (45) platforms for optimizing desired enzyme production. Looking ahead, utilizing agricultural residues or by-products as substrates for producing industrial enzymes via yeast fermentation could make the process more cost-effective.

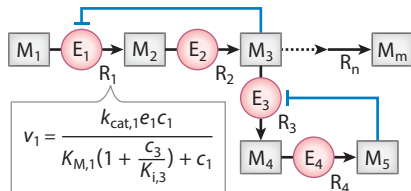
By 2050, conventional farming will be insufficient to provide 9 billion people with healthy food due to low conversion efficiency and the impacts of climate change. Yeast proteins, which are natural and sustainable, could play a crucial role in addressing this challenge. Companies such as Lesaffre (France) and Lallemand (Canada) have already commercialized yeast-based proteins as food-grade single-cell proteins (84). Furthermore, advancements in synthetic biology have facilitated the enhancement or modification of yeast proteins to meet the nutritional and sensory preferences of consumers. For instance, hemoglobin, an iron-binding protein essential for imparting a meat-like flavor, has been effectively produced by engineered yeast, achieving 18% of its intracellular protein as human hemoglobin (35). However, the application of yeast proteins as food products is currently limited by high production costs, technical challenges in extraction and refinement, and issues related to sensory qualities and palatability.

Peptides are short chains of amino acids and are generally smaller than proteins (65). Some peptides exhibit various beneficial effects on human or animal health, including antioxidant, antimicrobial, antihypertensive, antithrombotic, and immunomodulatory properties. Enzymatic hydrolysis and microbial fermentation of yeast have been used to produce bioactive peptides. For instance, small peptide fractions hydrolyzed from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein have been identified as antimicrobial peptides, which can be naturally secreted and produced by yeast (4). This discovery has guided the development of metabolic engineering strains for the production of GAPDH-derived peptides (3). In another study using an optimized expression system, two heterologous antimicrobial peptides, plantaricin 423 and mundticin ST4SA, were produced by engineered yeast, suggesting their potential as feed or food additives (85). Due to the uncontrollable nature of the autolysis process, the use of precise metabolic engineering strains is considered a promising approach for future developments.

## COMPUTATIONAL DESIGN OF METABOLISM

Mathematical modeling enables predictions of the cellular behavior in response to environmental and genetic perturbations, and therefore model-aided design and analysis of metabolism are expected to reduce the degree of trial and error and accelerate the design–build–test–learn cycle in metabolic engineering. Different mathematical models of metabolism have been used in the field of yeast metabolic engineering (**Figure 2**).

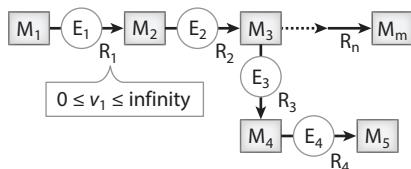
### Kinetic model



$$\begin{bmatrix} s_{11} & \dots & s_{1n} \\ \vdots & \ddots & \vdots \\ s_{m1} & \dots & s_{mn} \end{bmatrix} \cdot \begin{bmatrix} v_1 \\ \vdots \\ v_n \end{bmatrix} = \begin{bmatrix} \frac{dc_1}{dt} \\ \vdots \\ \frac{dc_m}{dt} \end{bmatrix}$$

$$v_i = f(k_{i,r}, e_{i,r}, c_j, \dots)$$

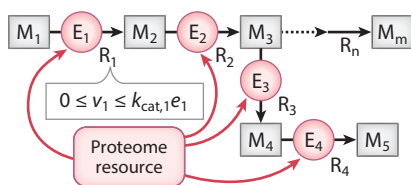
### Stoichiometric model



$$\begin{bmatrix} s_{11} & \dots & s_{1n} \\ \vdots & \ddots & \vdots \\ s_{m1} & \dots & s_{mn} \end{bmatrix} \cdot \begin{bmatrix} v_1 \\ \vdots \\ v_n \end{bmatrix} = \mathbf{0}$$

$$lb_i \leq v_i \leq ub_i$$

### Enzyme-constrained model



$$\begin{bmatrix} s_{11} & \dots & s_{1n} & 0 & \dots & 0 & 0 \\ \vdots & \ddots & \vdots & \vdots & \ddots & \vdots & \vdots \\ s_{m1} & \dots & s_{mn} & 0 & \dots & 0 & 0 \\ \frac{mw_1}{k_{cat,11}} & \dots & 0 & 1 & \dots & 0 & 0 \\ \vdots & \dots & \vdots & \vdots & \ddots & \vdots & \vdots \\ 0 & \dots & -\frac{mw_p}{k_{cat,pn}} & 0 & \dots & 1 & 0 \\ 0 & \dots & 0 & -1 & \dots & -1 & 1 \end{bmatrix} \cdot \begin{bmatrix} v_1 \\ \vdots \\ v_n \\ e_1 \\ \vdots \\ e_p \\ pool \end{bmatrix} = \mathbf{0}$$

$$lb_i \leq v_i \leq ub_i \quad -1,000 \leq e_j \leq 0 \quad -exp \leq pool \leq 0$$

Figure 2

Mathematical models of metabolism. Kinetic models describe individual reactions with detailed kinetic equations, which are a function of enzyme kinetic parameters, and concentrations of the enzyme, substrates, products, and regulators, if any. In addition, changes in metabolite concentrations over time are formulated on the basis of the rates of all the reactions in which the corresponding metabolites are involved. Stoichiometric models do not assign detailed kinetic equations to individual reactions but only impose lower and upper bounds on the reaction rates. On the basis of metabolite balancing as well, all reaction rates are formulated within systems of equations, in which the right-hand side of the equations are set to zero by assuming pseudo-steady state. Enzyme-constrained models are stoichiometric models integrated with enzyme constraints that are formulated on the basis of enzyme kinetics and concentrations. Likewise, enzyme-constrained models assume pseudo-steady state to reduce the computational burden.

## Kinetic Models

Kinetic models describe individual enzymatic reactions with rate law formalisms (56); i.e., the rate of an enzymatic reaction is determined by kinetic properties and the concentration of the enzyme and by concentrations of related metabolites, including not only the substrates and products of the reaction but also those regulating the enzyme (e.g., allosteric regulators). The rates of all reactions in the metabolic network are related by the change in the concentration of the shared metabolite over the change in time based on mass balance. Therefore, kinetic models allow for simulations of reaction rates and concentrations of metabolites and enzymes in a dynamic system.

In practice, kinetic models serve mostly as a computational framework to estimate the control on the metabolic system using the concept of metabolic control analysis (MCA) (22). MCA enables the identification of key reactions that should be modified, for example, by altering the expression of the enzyme level, in order to change the pathway flux or metabolite concentrations. This is done by calculating the so-called flux (or metabolite concentration) control coefficients, i.e., how

a fractional change in the flux (or metabolite concentration) is obtained following an infinitesimal fractional change in enzyme activity. Enzymes with large control coefficients play a dominant role in controlling the pathway flux and thus could be potential engineering targets for effectively improving the flux. For instance, MCA with the kinetic model of the glycerol synthesis pathway in *S. cerevisiae* quantified the control of enzymes in the pathway at the rate of glycerol production. It showed that glycerol 3-phosphate dehydrogenase had a flux control coefficient of approximately 0.85, while glycerol 3-phosphatase had a coefficient of 0.15 (15). This indicates that the former dominates the flux through glycerol synthesis and thus could be an efficient target for improving glycerol production. Besides the product synthesis, MCA can also be used to optimize the substrate uptake. An MCA study of co-utilization of glucose and xylose in *S. cerevisiae* (66) showed that the computed control coefficient of hexokinase over xylose uptake was  $-0.11$ , meaning that a twofold increase in the activity of hexokinase reduces xylose uptake by 11%. This finding indicates a negative impact of hexokinase on xylose uptake, validated by the experimental observation that *HXK2* deletion improves xylose uptake rate.

Despite the advantages of kinetic models (e.g., enabling mechanistic and dynamic simulations), there are few applications in yeast metabolic engineering due to some challenges and limitations. First, the construction of kinetic models requires prior knowledge and experimental data, particularly detailed rate law formalisms of all enzymatic reactions together with enzyme parameters. Because of data scarcity, current kinetic models either are at small scale or adopt many uncertain enzyme mechanisms and parameters, which could limit the predictive performance. Second, the computational costs and time of model construction and simulation are high. This is mainly due to the large systems of ordinary differential equations in the computational framework, which would further lead to a tremendous cost for model parameterization that depends on iterative simulations (94). Third, the effectiveness of MCA-based predictions might be limited. Traditional MCA studies have demonstrated that the control of a flux is distributed over several reactions (21); thus, there is in fact no rate-limiting reaction engineering that would significantly improve the flux (69).

## Stoichiometric Models

Different from kinetic models, pure stoichiometric models account for reaction stoichiometry while disregarding detailed rate law formalisms and thus are much more simplified in terms of mathematics. Stoichiometric models convert biochemical reactions into systems of ordinary differential equations based on mass balance as done in kinetic models, but usually adopt the assumption of pseudo-steady state to simplify the systems of equations, in which the variable is a vector of reaction rates and the coefficient matrix reflects reaction stoichiometry. The reaction rates can be determined by solving the systems of equations as linear programming problems with constraint-based approaches, typically flux balance analysis (FBA) (73). In addition, FBA optimizes a predefined reaction (i.e., objective function, subject to given constraints) and thus has the potential to predict optimal growth and productive capacity of cell factories. Notably, it takes a short time to run FBA (e.g., thousands of reactions within 1 s) (59).

Thanks to the simplified framework and high computational efficiency, stoichiometric models have been built for metabolism at the genome scale, resulting in genome-scale metabolic models (GEMs). Since the first GEM was built for *S. cerevisiae* in 2003 (20), an increasing number of GEMs have been developed, including not only those for various yeast species (17) but also multiple updated versions of the model for *S. cerevisiae* (11).

The yeast GEMs have been widely used in the field of metabolic engineering, and the applications published within the past 5 years involve prediction of engineering targets, identification of optimal pathways, FBA of cell factories, and optimization of culture conditions. First, most

studies have used GEMs to predict gene targets, engineering that could improve the flux toward the compound of interest. In silico knockout of genes can be performed with GEMs by either directly blocking the corresponding reactions or utilizing existing algorithms. For instance, OptGene is an evolutionary programming-based method that can rapidly identify gene deletion targets (75). Gene and reaction knockout predictions have led to effective targets for the overproduction of various compounds, such as L-phenylacetylcarbinol (33), 7-dehydrocholesterol (81), and rubusoside (104). GEMs have also been used to predict overexpression targets with the aid of design algorithms [e.g., FSEOF (flux scanning based on enforced objective flux) (14) and OptForce (82)]. These algorithms normally identify the reactions as overexpression targets whose rates increase with the product formation rate, leading to successful cases, including the overproduction of heme (34), taxadiene (60), and so on. Second, GEMs have allowed for comparison between pathways and thus guided pathway selection and design, which is based on the GEM-calculated pathway properties, especially the product yield on substrate. A recent example is the design of 3-hydroxypropionate overproduction in *S. cerevisiae*, in which the GEM suggested that targeting malonyl-CoA reductase into mitochondria could improve the theoretical yield (108). Third, FBA of cell factories also accounts for a large group of the applications, which aims to reveal the detailed mechanisms contributing to overproduction. Examples include FBA-based flux quantification for cell factories that overproduce  $\alpha$ -amylase (78), caffeic acid (7), and sclareol (5). Last, GEMs have been used to investigate and optimize the parameters of culture conditions [e.g., pH (24) and inlet air flow (64)] of *S. cerevisiae* for ethanol production.

With the extensive applications in metabolic engineering, limitations of GEMs have emerged. Current GEM-guided strategies depend exclusively on the optimization of substrate utilization as the objective, which, however, conflicts with the finding that cells are simultaneously subject to multiple objectives and constraints (86) and thus limits the predictive performance. To address this issue, efforts have focused on integration of biological constraints into the stoichiometric modeling framework (12, 89).

### Enzyme-Constrained Models

Enzyme-constrained models (ecModels) are stoichiometric models integrated with enzyme kinetics. The core of ecModels is to couple the usage of the enzyme to the corresponding metabolic reaction on the basis of the equation  $v \leq k_{\text{cat}}e$ , in which  $v$  is the reaction rate,  $k_{\text{cat}}$  is the enzyme turnover number, and  $e$  is the enzyme concentration. With this, ecModels can account for the impact of enzyme concentrations (i.e., proteome resource) on cellular behaviors and thus outperform the conventional GEMs. With the development of ecModel construction toolboxes [e.g., GECKO (9) and ECMpy (62)], ecModels have been built for various organisms (39). Moreover, by implementing deep learning prediction of  $k_{\text{cat}}$  values (46), the latest GECKO toolbox claims that it is now possible to build ecModels for any organism (9).

While ecModels have been built for more than 300 yeast species in a large-scale manner (46), the ecModels for *S. cerevisiae* have gained more attention than models for other species have. In a recent study, the *S. cerevisiae* ecModel was used to calculate the costs of substrate and protein resources for synthesizing a metabolite. The substrate cost indicates how many units of substrate (e.g., glucose) are required for the synthesis of one unit of the metabolite, while the protein cost calculates the protein mass required per unit of the metabolite biosynthetic flux (13). By calculating the glucose and protein costs for the biosynthesis of the 20 proteinogenic amino acids in *S. cerevisiae*, the study identified a weak correlation between the two types of costs and found that protein cost rather than glucose cost could explain the amino acid composition in yeast. Thus, this study demonstrates the potential effectiveness of ecModels in guiding engineering strategies, and

the finding highlights the importance of considering the proteome resource when designing cell factories.

In addition to the enzyme constraint, other biological constraints have been investigated. One example integrated the enzyme cofactors (i.e., metal ions) into the *S. cerevisiae* GEM by formulating protein synthesis and enzyme cofactor binding reactions (10). The resulting model, named CofactorYeast, coupled the rates of metabolic reactions with the synthesis rates of the corresponding enzymes and also the metal usage. Therefore, CofactorYeast can predict cellular behaviors upon metal limitation (e.g., decreased production of *p*-coumaric acid in response to iron depletion) (10). Another example formulated the complete protein secretory pathway within the *S. cerevisiae* GEM, which enabled the researchers to account for the resource of secretory machineries and also to calculate the secretory cost of a protein (45). The resulting model, named pcSecYeast, accelerated hypothesis generation for phenotypes that might be caused by limited secretory capacity. In addition, pcSecYeast predicted targets for overproduction of recombinant proteins, and many of those for  $\alpha$ -amylase overproduction were experimentally validated in the study (45). These studies demonstrate that metabolism is under the control of multiple constraints, and future modeling efforts could integrate more biological constraints and processes within a unified framework.

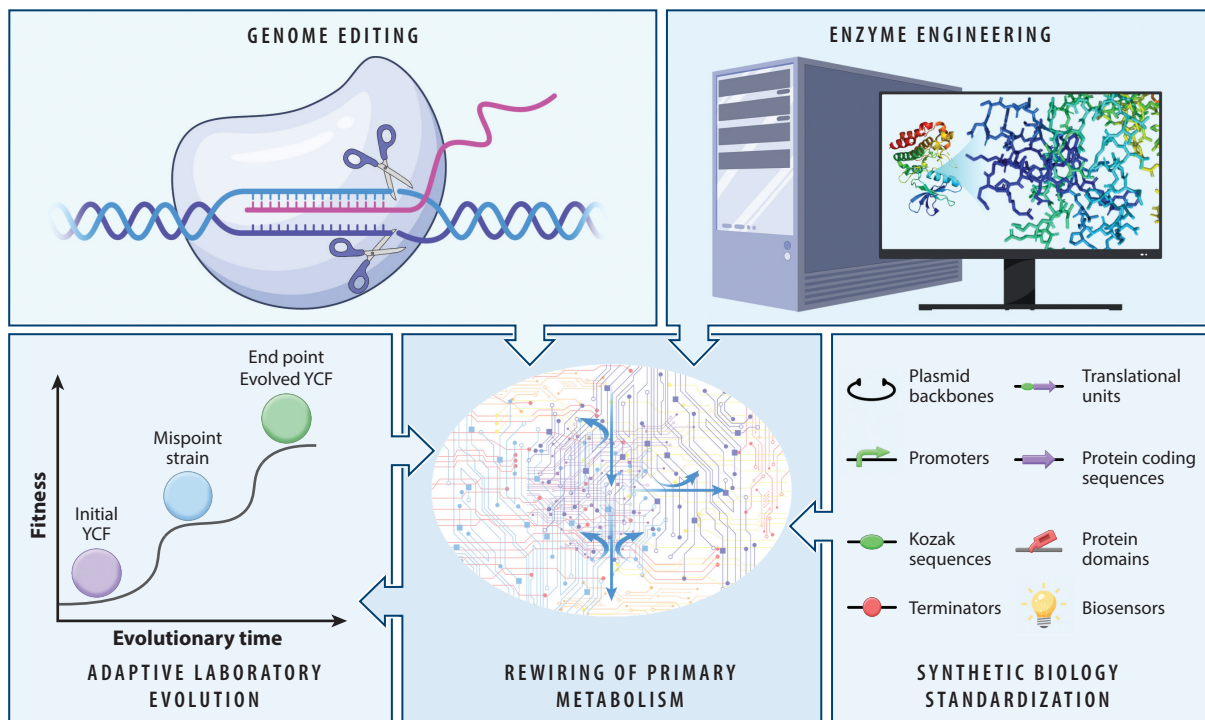
## ENGINEERING METABOLISM

Yeast is not inherently optimized for producing any chemical of interest. Developing an engineered yeast strain therefore necessitates extensive modifications to the host cell's metabolism. These modifications include adjusting expression levels, performing gene knockouts, and implementing enzyme engineering strategies. Recent advancements in synthetic biology tools have significantly enhanced our ability to elucidate complex phenotypes and engineer novel ones for sophisticated biological applications (Figure 3).

### Genome Editing

Genome editing technology is a powerful tool for metabolic engineering by enabling precise gene modifications in living cells. Utilizing genome editing tools allows for the alteration of metabolic pathways and the control over cellular metabolism through targeted gene modifications.

The advanced clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) protein genome editing system has significantly enhanced strain construction, offering faster and more reliable methods by facilitating genetic integration, deletion, or replacement via intrinsic DNA repair mechanisms. Meanwhile, enhanced functionalities, such as transcriptional regulation, methylation, and base editing, have been achieved by fusing CRISPR-Cas with functional effectors like transcriptional effectors, methyltransferases, and deaminases (88). The development of a CRISPR-Cas system with orthogonal functions has proven to be a versatile and powerful tool for implementing combinatorial and multiplex modifications essential for creating superior yeast cell factories. For instance, the GTR-CRISPR system (a gRNA-tRNA array for CRISPR-Cas9) has enabled the multiplexed engineering of the yeast lipid network, simultaneously disrupting eight lipid-related genes with 87% efficiency and resulting in a 30-fold increase in free fatty acid production (109). Additionally, the use of three orthogonal Cas proteins to construct a trifunctional CRISPR system has facilitated simultaneous transcriptional activation, interference, and gene deletion, tripling  $\beta$ -carotene production in a single step (49). This approach was further enhanced by integrating array-synthesized oligo pools for genome-scale genotype-phenotype mapping, identifying previously unknown genetic determinants of complex traits such as furfural tolerance (50). Genome-wide or combinatorial CRISPR engineering techniques substantially advance genotype-phenotype mapping, facilitate the construction of complex biological



**Figure 3**

Schematic overview of synthetic biology tools and strategies to implement the ideal yeast cell factory (YCF). The figure illustrates various methods used to engineer yeast metabolism, facilitating the construction and optimization of efficient YCFs. Key tools and strategies include genome editing, enzyme engineering, adaptive laboratory evolution, and synthetic biology standardization.

systems, and enhance the metabolic engineering of yeast cell factories. However, a critical factor in these approaches is the availability of high-throughput screening or selection strategies that efficiently identify desired phenotypes.

In addition to the well-known CRISPR-Cas system, several alternative genome editing technologies have been developed for yeast. For instance, transcription activator-like effector nucleases (TALENs) were the first accessible genome editing technology, and a TALEN-assisted method has been developed to enhance yeast ethanol tolerance (23). The primary limitations of TALEN-based techniques are their reliance on protein–DNA interactions and the necessity to design new proteins for each specific application. In yeast with synthetic chromosomes, symmetrical *loxP* sites are introduced along with Cre recombinase as the controller, enabling various recombination events. This method, known as synthetic chromosome rearrangement and modification by *loxP*sym-mediated evolution (SCRaMbLE), has proven effective in inducing genome rearrangement and accelerating phenotypic evolution (37). However, the SCRaMbLE system cannot facilitate rational genomic changes and its use is restricted to synthetic yeast. Concurrently, a nuclease-independent eukaryotic multiplex automated genome engineering (eMAGE) platform for yeast has been introduced (2). eMAGE employs single-stranded oligonucleotides to swiftly generate precise combinatorial genome modifications, enabling up to 60 targeted mutations in a single transformation. It has been effectively utilized for multiplex editing and optimization of a heterologous  $\beta$ -carotene biosynthetic pathway. However, the efficient multiplex editing of eMAGE is restricted to a region of approximately 20 kb surrounding the selectable marker.

## Enzyme Engineering

Enzymes are fundamental to the design and construction of engineered yeast systems. However, native enzymes that exhibit limitations such as low activity, low stability, and susceptibility to feedback inhibition may not meet specific requirements. In such instances, protein engineering can be utilized to develop modified enzymes with altered functions. Common protein engineering techniques include directed evolution and rational engineering.

Rational engineering relies on a thorough understanding of the enzyme function and mechanism, derived from existing protein structures or homology models. For instance, rational design has identified residues critical for isomeric substrate selectivity in monoterpene synthases, facilitating the engineering of five different enzymes to accept an alternative substrate with increased efficiency and specificity, leading to the synthesis of 134.8 mg/L of limonene and 72.4 mg/L of sabinene (32). Another promising approach involves the design of de novo artificial enzymes using computational methods (95), although this strategy has yet to be applied in yeast.

Directed evolution, which simulates natural selection, does not require protein structural information, allowing its application to any enzyme. This method necessitates a suitable high-throughput assay or a connection to growth or pigment synthesis. For example, directed evolution of isoprene synthase, driven by precursor toxicity, achieved enhanced catalytic activity, with the best mutant producing 3.7 g/L of isoprene (98). Although rational engineering is significantly developed, directed evolution remains powerful, as it often reveals beneficial mutations at unexpected sites. Future applications might include the use of electrochemical sensors or biosensors to link yeast performance with observable signals or cell growth, thus enhancing high-throughput screening capabilities.

Semirational design, a hybrid of rational protein design and directed evolution, balances sequence diversity and workload. It generates a small but smart mutant library for directed evolution, guided by *in silico* models. For example, in constructing a yeast cell factory for ginsenoside Rh2, semirational design improved the catalytic efficiency of the natural glycosyltransferase UGT51 by approximately 1,800-fold (114). Introducing this mutant resulted in a 122-fold increase in Rh2 production in yeast. When combined with other metabolic engineering strategies, the final strain produced approximately 300 mg/L of Rh2. Instead of focusing on one or two key amino acids, future approaches of semirational design could target mutagenesis to specific protein regions to introduce significant sequence changes, thereby facilitating the creation of beneficial variants with increasingly ambitious goals.

## Adaptive Laboratory Evolution

ALE utilizes natural selection to optimize biological systems by forcing the selection of specific phenotypes through the accumulation of spontaneous mutations under constant selection pressure. An effective selection strategy is crucial for successful ALE. The classical growth-coupling strategy is commonly used, exemplified by ALE enhancing the utilization of nonpreferred methanol as a substrate (19), balancing metabolic flux (79), and increasing tolerance for toxic aromatic acids (76). However, desired phenotypes are not always associated with growth.

To address this, researchers have employed genetic biosensors to facilitate ALE for phenotypes that do not directly benefit growth, such as high production of metabolites. In recent research, a synthetic selection system was developed that links *cis,cis*-muconic acid (CCM) production to cell fitness via a CCM biosensor (91). Multiple rounds of mutagenesis and ALE identified a strain producing 2.1 g/L of muconic acid. Currently, the number of available biosensors is limited, highlighting the need to expand the range of detectable metabolites and factors.

Alternatively, a tailor-made growth-coupling strategy, though less universal, has also been utilized to support ALE. For example, the use of H<sub>2</sub>O<sub>2</sub> has driven the ALE of astaxanthin-producing yeast, where intracellular astaxanthin provides fitness advantages under oxidative stress (38). This approach led to the highest reported titer of 404.78 mg/L astaxanthin in yeast. However, this strategy is highly specific and relies on a deep understanding of the desired trait's properties, such as the antioxidant capacity of astaxanthin.

After ALE, employing systems biology and machine learning techniques can further elucidate the genetic basis of the adaptation process. This knowledge aids in designing more robust microbial strains for industrial bioprocesses through a reverse engineering approach (19, 76, 79). Additionally, integrating ALE with rational genetic engineering could maximize the power. Using a rationally engineered parental microbe as the starter can expedite the evolution process, particularly for complex phenotypes (79, 105).

### **Synthetic Biology Standardization**

Standards form the foundation of synthetic biology technology and involve the precise description and measurement of basic elements, such as promoters/terminators of varying strengths, coding sequences with distinct functions, and regulatory sequences for complex genetic circuits. These elements can be assembled to create devices or potentially entire synthetic biological systems that operate in specific ways. For instance, a library of synthetic core promoters has been developed and validated as modular parts for fine-tuning gene expression (77). Another notable example of standardization is the design and construction of TALE (transcription activator-like effector) constructs, which serve as a genetic circuit for producing zinc finger DNA recognition domains utilized in TALEN genome editing tools (6). Currently, several collections of characterized yeast elements are available, including the iGEM Registry (31) and the Joint Bioenergy Institute's Inventory of Composable Elements (27). Additionally, novel standardization parameters such as loci characterization, DNA integration efficiency, and newly identified elements with novel functions are continually being added to these toolkits (63, 106).

Meanwhile, various tools and techniques are available for assembling DNA or synthetic biology elements, with the choice of method depending on the specific application. For example, MoClo, based on the Golden Gate assembly method, enables a rapid and modular assembly for multiparts (101). YeastFab facilitates the combinatorial assembly of pathways using hundreds of regulatory biological parts through the incorporation of prefixes and suffixes (26). However, manual approaches to assembling large combinations of parts using these tools are laborious, repetitive, error-prone, and time-consuming. Therefore, the suitability of individual toolkits for automation should be further evaluated in terms of cost-effectiveness and time savings (6, 74).

### **FUTURE PERSPECTIVES**

To date, yeast has been engineered as a versatile platform host for a diverse array of products thanks to its well-characterized genome, beneficial traits, and advanced genetic toolkit. Commercial-scale plants utilizing engineered yeast strains are already operational or in development for the production of biofuels (36), such as second-generation ethanol by companies like DuPont, POET-DSM, and GranBio; chemicals (18), including succinic acid by DSM, isobutanol by Gevo, and farnesene by Amyris; biopharmaceuticals (96), such as insulin by Novo Nordisk and Vaxelis by Merck; and natural molecules (97), including resveratrol and nootkatone by Evolva. Recent advances in microbial fermentation aim not only to maximize the TRY of desired products but also to adapt microbial catabolism for the use of cost-effective feedstocks (54, 111). For instance, carbon-neutral or carbon-negative production is achievable through microbial biosynthesis from C1 feedstocks,

with electricity providing the energy needed for microbial catalysis to convert CO<sub>2</sub> into fuels and other organic commodities.

The eukaryotic nature of yeast enables it to perform posttranslational modifications similar to those in plants. Yeast cells are also equipped with subcellular organelles like the endoplasmic reticulum and mitochondria, which are essential for enzyme localization or protection. These traits make yeast suitable for producing a variety of complex natural products, providing a significant advantage over bacteria. Additionally, yeasts offer significant advantages over higher eukaryotes such as mammalian or plant cells due to their rapid growth and ability to achieve high cell densities. These characteristics make yeasts particularly suitable for large-scale production, leading to higher yields of the desired plant-based natural products. Indeed, a myriad of plant metabolites (68), including terpenes, flavonoids, alkaloids, and betalains, have been synthesized in yeast. With the aid of synthetic biology tools, such as CRISPR-Cas, combinatorial optimization techniques, and artificial intelligence-aided protein engineering, yeasts have been metabolically engineered to produce even more complex plant-based natural products (52, 107).

Metabolic models are powerful tools that offer profound insights into cellular metabolism, facilitating the rational design and optimization of cell factories for the production of a diverse array of bioproducts. Different types of models have distinct applications. Kinetic models enable quantitative identification of the key nodes controlling fluxes through pathways or networks, and GEMs and ecModels predict design strategies based on the optimization of resources (e.g., substrate and proteome). It should be noted that ecModels appear to be a kinetic–stoichiometric hybrid, and the hybrid does not increase computational cost too much but allows for MCA at the genome scale (57). Therefore, more elements of kinetic models are expected to be introduced into the stoichiometric modeling framework to improve model performance. The construction of the hybrid models may be limited by the lack of genome-scale parameters, which could be addressed by machine learning and deep learning algorithms with biofoundry-based data generation.

Future engineered yeasts can produce molecules that are currently unattainable through synthetic chemistry. The use of artificial enzymes, as demonstrated in the development of nonnatural cyclopropanated terpenoids (29), is expected to expand the enzymatic toolbox, facilitating critical chemical reactions. Additionally, incorporating artificial amino acids into proteins deserves special attention, as it can enhance the diversity of protein functions for industrial applications. Furthermore, multiplexed automated culture platforms facilitate the development of desired yeast strains by enabling more precise control over selection, increasing the number of independent replicates, and reducing labor costs (102).

In conclusion, yeast cell factories provide a combination of genetic tractability, high yield potential, scalability, cost-effectiveness, and sustainability. These attributes make them an attractive platform for the production of various compounds. With ongoing advancements in various tools and techniques, metabolic engineering aimed at rewiring cellular metabolism is rapidly evolving, further expanding the industrial applications for yeast cell factories.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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## LITERATURE CITED

1. Baptista SL, Costa CE, Cunha JT, Soares PO, Domingues L. 2021. Metabolic engineering of *Saccharomyces cerevisiae* for the production of top value chemicals from biorefinery carbohydrates. *Biotechnol. Adv.* 47:107697
2. Barbieri EM, Muir P, Akhuetie-Oni BO, Yellman CM, Isaacs FJ. 2017. Precise editing at DNA replication forks enables multiplex genome engineering in eukaryotes. *Cell* 171:1453–67.e13
3. Branco P, Carvalho L, Prista C, Albergaria H. 2023. Effect of overexpression of partial *TDH1* and *TDH2/3* gene sequences in a starter strain of industrial bioethanol fermentation on the *Brettanomyces bruxellensis* contaminant growth. *Lett. Appl. Microbiol.* 76:ovad141
4. Branco P, Francisco D, Chambon C, Hébraud M, Arneborg N, et al. 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* 98:843–53
5. Cao X, Yu W, Chen Y, Yang S, Zhao ZK, et al. 2023. Engineering yeast for high-level production of diterpenoid sclareol. *Metab. Eng.* 75:19–28
6. Chao R, Liang J, Tasan I, Si T, Ju L, Zhao H. 2017. Fully automated one-step synthesis of single-transcript TALEN pairs using a biological foundry. *ACS Synth. Biol.* 6:678–85
7. Chen R, Gao J, Yu W, Chen X, Zhai X, et al. 2022. Engineering cofactor supply and recycling to drive phenolic acid biosynthesis in yeast. *Nat. Chem. Biol.* 18:520–29
8. Chen S, Xu Z, Ding B, Zhang Y, Liu S, et al. 2023. Big data mining, rational modification, and ancestral sequence reconstruction inferred multiple xylose isomerases for biorefinery. *Sci. Adv.* 9:eadd8835
9. Chen Y, Gustafsson J, Tafur Rangel A, Anton M, Domenzain I, et al. 2024. Reconstruction, simulation and analysis of enzyme-constrained metabolic models using GECKO Toolbox 3.0. *Nat. Protoc.* 19:629–67
10. Chen Y, Li F, Mao J, Chen Y, Nielsen J. 2021. Yeast optimizes metal utilization based on metabolic network and enzyme kinetics. *PNAS* 118:e2020154118
11. Chen Y, Li F, Nielsen J. 2022. Genome-scale modeling of yeast metabolism: retrospectives and perspectives. *FEMS Yeast Res.* 22:foac003
12. Chen Y, Nielsen J. 2021. Mathematical modeling of proteome constraints within metabolism. *Curr. Opin. Syst. Biol.* 25:50–56
13. Chen Y, Nielsen J. 2022. Yeast has evolved to minimize protein resource cost for synthesizing amino acids. *PNAS* 119:e2114622119
14. Choi HS, Lee SY, Kim TY, Woo HM. 2010. In silico identification of gene amplification targets for improvement of lycopene production. *Appl. Environ. Microbiol.* 76:3097–105
15. Cronwright GR, Rohwer JM, Prior BA. 2002. Metabolic control analysis of glycerol synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 68:4448–56
16. Crook N, Sun J, Morse N, Schmitz A, Alper HS. 2016. Identification of gene knockdown targets conferring enhanced isobutanol and 1-butanol tolerance to *Saccharomyces cerevisiae* using a tunable RNAi screening approach. *Appl. Microbiol. Biotechnol.* 100:10005–18
17. Domenzain I, Li F, Kerkhoven EJ, Siewers V. 2021. Evaluating accessibility, usability and interoperability of genome-scale metabolic models for diverse yeasts species. *FEMS Yeast Res.* 21:foab002
18. Ekas H, Deaner M, Alper HS. 2019. Recent advancements in fungal-derived fuel and chemical production and commercialization. *Curr. Opin. Biotechnol.* 57:1–9
19. Espinosa MI, Gonzalez-Garcia RA, Valgepea K, Plan MR, Scott C, et al. 2020. Adaptive laboratory evolution of native methanol assimilation in *Saccharomyces cerevisiae*. *Nat. Commun.* 11:5564
20. Förster J, Famili I, Fu P, Palsson B, Nielsen J. 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. *Genome Res.* 13:244–53
21. Fell D. 1997. *Understanding the Control of Metabolism*: London: Portland Press
22. Fell DA. 2021. Metabolic control analysis. In *Metabolic Engineering: Concepts and Applications*, ed. J Nielsen, G Stephanopoulos, SY Lee, pp. 171–211. Weinheim, Ger.: Wiley
23. Gan Y, Lin Y, Guo Y, Qi X, Wang Q. 2018. Metabolic and genomic characterisation of stress-tolerant industrial *Saccharomyces cerevisiae* strains from TALENs-assisted multiplex editing. *FEMS Yeast Res.* 18:foy045

24. Ghaffarinasab S, Motamedian E. 2021. Improving ethanol production by studying the effect of pH using a modified metabolic model and a systemic approach. *Biotechnol. Bioeng.* 118:2934–46
25. Guo J, Suástegui M, Sakimoto KK, Moody VM, Xiao G, et al. 2018. Light-driven fine chemical production in yeast biohybrids. *Science* 362:813–16
26. Guo Y, Dong J, Zhou T, Auxillos J, Li T, et al. 2015. YeastFab: the design and construction of standard biological parts for metabolic engineering in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 43:e88
27. Ham TS, Dmytriv Z, Plahar H, Chen J, Hillson NJ, Keasling JD. 2012. Design, implementation and practice of JBEI-ICE: an open source biological part registry platform and tools. *Nucleic Acids Res.* 40:e141
28. Hou J, Tyo K, Liu Z, Petranovic D, Nielsen J. 2012. Engineering of vesicle trafficking improves heterologous protein secretion in *Saccharomyces cerevisiae*. *Metab. Eng.* 14:120–27
29. Huang J, Liu Z, Bloomer BJ, Clark DS, Mukhopadhyay A, et al. 2021. Unnatural biosynthesis by an engineered microorganism with heterologously expressed natural enzymes and an artificial metalloenzyme. *Nat. Chem.* 13:1186–91
30. Huang M, Wang G, Qin J, Petranovic D, Nielsen J. 2018. Engineering the protein secretory pathway of *Saccharomyces cerevisiae* enables improved protein production. *PNAS* 115:E11025
31. iGEM. Yeast. *iGEM*. <http://parts.igem.org/Yeast>
32. Ignea C, Raadam MH, Motawia MS, Makris AM, Vickers CE, Kampranis SC. 2019. Orthogonal monoterpenoid biosynthesis in yeast constructed on an isomeric substrate. *Nat. Commun.* 10:3799
33. Iranmanesh E, Asadollahi MA, Biria D. 2020. Improving L-phenylacetylcarbinol production in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. *J. Biotechnol.* 308:27–34
34. Ishchuk OP, Domenzain I, Sánchez BJ, Muñiz-Paredes F, Martínez JL, et al. 2022. Genome-scale modeling drives 70-fold improvement of intracellular heme production in *Saccharomyces cerevisiae*. *PNAS* 119:e2108245119
35. Ishchuk OP, Frost AT, Muñiz-Paredes F, Matsumoto S, Laforge N, et al. 2021. Improved production of human hemoglobin in yeast by engineering hemoglobin degradation. *Metab. Eng.* 66:259–67
36. Jansen ML, Bracher JM, Papapetridis I, Verhoeven MD, de Bruijn H, et al. 2017. *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. *FEMS Yeast Res.* 17:fox044
37. Jia B, Wu Y, Li B-Z, Mitchell LA, Liu H, et al. 2018. Precise control of SCRaMble in synthetic haploid and diploid yeast. *Nat. Commun.* 9:1933
38. Jiang G, Yang Z, Wang Y, Yao M, Chen Y, et al. 2020. Enhanced astaxanthin production in yeast via combined mutagenesis and evolution. *Biochem. Eng. J.* 156:107519
39. Kerkhoven EJ. 2022. Advances in constraint-based models: methods for improved predictive power based on resource allocation constraints. *Curr. Opin. Microbiol.* 68:102168
40. King AB. 2009. Insulin detemir: a better basal insulin for the management of diabetes? *Exp. Rev. Endocrinol. Metab.* 4:15–23
41. Kjeldsen T, Andersen AS, Hubálek F, Johansson E, Kreiner FF, et al. 2024. Molecular engineering of insulin for recombinant expression in yeast. *Trends Biotechnol.* 42:464–78
42. Kong X, Wu Y, Yu W, Liu Y, Li J, et al. 2023. Efficient synthesis of limonene in *Saccharomyces cerevisiae* using combinatorial metabolic engineering strategies. *J. Agric. Food Chem.* 71:7752–64
43. Konzock O, Nielsen J. 2024. TRYing to evaluate production costs in microbial biotechnology. *Trends Biotechnol.* 42:1339–47
44. Kumari P, Sharma J, Singh AK, Pandey AK, Yusuf F, et al. 2023. Tailored designing of a diploid *S. cerevisiae* natural isolate for increased production of fatty acid ethyl ester. *Chem. Eng. J.* 453:139852
45. Li F, Chen Y, Qi Q, Wang Y, Yuan L, et al. 2022. Improving recombinant protein production by yeast through genome-scale modeling using proteome constraints. *Nat. Commun.* 13:2969
46. Li F, Yuan L, Lu H, Li G, Chen Y, et al. 2022. Deep learning-based  $k_{cat}$  prediction enables improved enzyme-constrained model reconstruction. *Nat. Catal.* 5:662–72
47. Li H, Ma W, Wang W, Gao S, Shan X, Zhou J. 2024. Synergetic engineering of multiple pathways for de novo (2S)-naringenin biosynthesis in *Saccharomyces cerevisiae*. *ACS Sustain. Chem. Eng.* 12:59–71
48. Li M, Zhou P, Chen M, Yu H, Ye L. 2022. Spatiotemporal regulation of astaxanthin synthesis in *S. cerevisiae*. *ACS Synth. Biol.* 11:2636–49

49. Lian J, Hamedirad M, Hu S, Zhao H. 2017. Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system. *Nat. Commun.* 8:1688
50. Lian J, Schultz C, Cao M, Hamedirad M, Zhao H. 2019. Multi-functional genome-wide CRISPR system for high throughput genotype–phenotype mapping. *Nat. Commun.* 10:5794
51. Liu H, Zhou P, Qi M, Guo L, Gao C, et al. 2022. Enhancing biofuels production by engineering the actin cytoskeleton in *Saccharomyces cerevisiae*. *Nat. Commun.* 13:1886
52. Liu Y, Zhao X, Gan F, Chen X, Deng K, et al. 2024. Complete biosynthesis of QS-21 in engineered yeast. *Nature* 629:937–44
53. Liu Z, Tyo KEJ, Martínez JL, Petranovic D, Nielsen J. 2012. Different expression systems for production of recombinant proteins in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 109:1259–68
54. Liu Z, Wang K, Chen Y, Tan T, Nielsen J. 2020. Third-generation biorefineries as the means to produce fuels and chemicals from CO<sub>2</sub>. *Nat. Catal.* 3:274–88
55. Lopes ML, de Lima Paulillo SC, Godoy A, Cherubin RA, Lorenzi MS, et al. 2016. Ethanol production in Brazil: a bridge between science and industry. *Braz. J. Microbiol.* 47:64–76
56. Lu H, Chen Y, Nielsen J, Kerkhoven EJ. 2021. Kinetic models of metabolism. In *Metabolic Engineering: Concepts and Applications*, ed. J Nielsen, G Stephanopoulos, SY Lee, pp. 153–70. Weinheim, Ger.: Wiley
57. Lu H, Li F, Yuan L, Domenzain I, Yu R, et al. 2021. Yeast metabolic innovations emerged via expanded metabolic network and gene positive selection. *Mol. Syst. Biol.* 17:e10427
58. Luo X, Reiter MA, d’Espaux L, Wong J, Denby CM, et al. 2019. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. *Nature* 567:123–26
59. Machado D. 2024. A benchmark of optimization solvers for genome-scale metabolic modeling of organisms and communities. *mSystems* 9:e0083323
60. Malci K, Santibáñez R, Jonguitud-Borrego N, Santoyo-García JH, Kerkhoven EJ, Rios-Solis L. 2023. Improved production of Taxol® precursors in *S. cerevisiae* using combinatorial in silico design and metabolic engineering. *Microb. Cell Fact.* 22:243
61. Malik K, Sharma P, Yang Y, Zhang P, Zhang L, et al. 2022. Lignocellulosic biomass for bioethanol: insight into the advanced pretreatment and fermentation approaches. *Ind. Crops Prod.* 188:115569
62. Mao Z, Niu J, Zhao J, Huang Y, Wu K, et al. 2024. ECMpy 2.0: a Python package for automated construction and analysis of enzyme-constrained models. *Synth. Syst. Biotechnol.* 9:494–502
63. Meng J, Qiu Y, Zhang Y, Zhao H, Shi S. 2023. CMI: CRISPR/Cas9 based efficient multiplexed integration in *Saccharomyces cerevisiae*. *ACS Synth. Biol.* 12:1408–14
64. Mesquita TJB, Campani G, Giordano RC, Zangirolami TC, Horta ACL. 2021. Machine learning applied for metabolic flux-based control of micro-aerated fermentations in bioreactors. *Biotechnol. Bioeng.* 118:2076–91
65. Mirzaei M, Shavandi A, Mirdamadi S, Soleymanzadeh N, Motahari P, et al. 2021. Bioactive peptides from yeast: a comparative review on production methods, bioactivity, structure-function relationship, and stability. *Trends Food Sci. Technol.* 118:297–315
66. Miskovic L, Alff-Tuomala S, Soh KC, Barth D, Salusjärvi L, et al. 2017. A design–build–test cycle using modeling and experiments reveals interdependencies between upper glycolysis and xylose uptake in recombinant *S. cerevisiae* and improves predictive capabilities of large-scale kinetic models. *Biotechnol. Biofuels Bioprod.* 10:166
67. MohammadiPeyhani H, Hafner J, Sveshnikova A, Viterbo V, Hatzimanikatis V. 2022. Expanding biochemical knowledge and illuminating metabolic dark matter with ATLASx. *Nat. Commun.* 13:1560
68. Naseri G. 2023. A roadmap to establish a comprehensive platform for sustainable manufacturing of natural products in yeast. *Nat. Commun.* 14:1916
69. Niederberger P, Prasad R, Miozzari G, Kacser H. 1992. A strategy for increasing an in vivo flux by genetic manipulations. The tryptophan system of yeast. *Biochem. J.* 287(Part 2):473–79
70. Nielsen J. 2019. Yeast systems biology: model organism and cell factory. *Biotechnol. J.* 14:1800421
71. Nielsen J, Keasling JD. 2016. Engineering cellular metabolism. *Cell* 164:1185–97
72. Nielsen J, Larsson C, van Maris A, Pronk J. 2013. Metabolic engineering of yeast for production of fuels and chemicals. *Curr. Opin. Biotechnol.* 24:398–404
73. Orth JD, Thiele I, Palsson B. 2010. What is flux balance analysis? *Nat. Biotechnol.* 28:245–48

74. Otero-Muras I, Carbonell P. 2021. Automated engineering of synthetic metabolic pathways for efficient biomanufacturing. *Metab. Eng.* 63:61–80
75. Patil KR, Rocha I, Förster J, Nielsen J. 2005. Evolutionary programming as a platform for in silico metabolic engineering. *BMC Bioinform.* 6:308
76. Pereira R, Mohamed ET, Radi MS, Herrgård MJ, Feist AM, et al. 2020. Elucidating aromatic acid tolerance at low pH in *Saccharomyces cerevisiae* using adaptive laboratory evolution. *PNAS* 117:27954–61
77. Portela RMC, Vogl T, Kniely C, Fischer JE, Oliveira R, Glieder A. 2017. Synthetic core promoters as universal parts for fine-tuning expression in different yeast species. *ACS Synth. Biol.* 6:471–84
78. Qi Q, Li F, Yu R, Engqvist MKM, Siewers V, et al. 2020. Different routes of protein folding contribute to improved protein production in *Saccharomyces cerevisiae*. *mBio* 11:e02743-20
79. Qin N, Li L, Ji X, Pereira R, Chen Y, et al. 2023. Flux regulation through glycolysis and respiration is balanced by inositol pyrophosphates in yeast. *Cell* 186:748–63.e15
80. Qin N, Li L, Wan X, Ji X, Chen Y, et al. 2024. Increased CO<sub>2</sub> fixation enables high carbon-yield production of 3-hydroxypropionic acid in yeast. *Nat. Commun.* 15:1591
81. Qu L, Xiu X, Sun G, Zhang C, Yang H, et al. 2022. Engineered yeast for efficient de novo synthesis of 7-dehydrocholesterol. *Biotechnol. Bioeng.* 119:1278–89
82. Ranganathan S, Suthers PF, Maranas CD. 2010. OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS Comput. Biol.* 6:e1000744
83. Rendulić T, Perpelea A, Ortiz JPR, Casal M, Nevoigt E. 2024. Mitochondrial membrane transporters as attractive targets for the fermentative production of succinic acid from glycerol in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 24:foae009
84. Ritala A, Häkkinen ST, Toivari M, Wiebe MG. 2017. Single cell protein—state-of-the-art, industrial landscape and patents 2001–2016. *Front. Microbiol.* 8:2009
85. Rossouw M, Cripwell RA, Vermeulen RR, van Staden AD, van Zyl WH, et al. 2024. Heterologous expression of plantaricin 423 and mundticin ST4SA in *Saccharomyces cerevisiae*. *Probiotics Antimicrob. Proteins* 16:845–61
86. Schuetz R, Zamboni N, Zampieri M, Heinemann M, Sauer U. 2012. Multidimensional optimality of microbial metabolism. *Science* 336:601–4
87. Shi S, Chen Y, Siewers V, Nielsen J. 2014. Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. *mBio* 5:e01130-14
88. Shi S, Qi N, Nielsen J. 2022. Microbial production of chemicals driven by CRISPR-Cas systems. *Curr. Opin. Biotechnol.* 73:34–42
89. Shi Z, Liu P, Liao X, Mao Z, Zhang J, et al. 2022. Data-driven synthetic cell factories development for industrial biomanufacturing. *Biodesign Res.* 2022:9898461
90. Si T, Luo Y, Xiao H, Zhao H. 2014. Utilizing an endogenous pathway for 1-butanol production in *Saccharomyces cerevisiae*. *Metab. Eng.* 22:60–68
91. Snoek T, Romero-Suarez D, Zhang J, Ambri F, Skjoedt ML, et al. 2018. An orthogonal and pH-tunable sensor-selector for muconic acid biosynthesis in yeast. *ACS Synth. Biol.* 7:995–1003
92. Srinivasan P, Smolke CD. 2021. Engineering cellular metabolite transport for biosynthesis of computationally predicted tropane alkaloid derivatives in yeast. *PNAS* 118:e2104460118
93. Tang M-C, Shen C, Deng Z, Ohashi M, Tang Y. 2022. Combinatorial biosynthesis of terpenoids through mixing-and-matching sesquiterpene cyclase and cytochrome P450 pairs. *Org. Lett.* 24:4783–87
94. Tummler K, Klipp E. 2018. The discrepancy between data for and expectations on metabolic models: how to match experiments and computational efforts to arrive at quantitative predictions? *Curr. Opin. Syst. Biol.* 8:1–6
95. Vázquez Torres S, Leung PJ, Venkatesh P, Lutz ID, Hink F, et al. 2024. De novo design of high-affinity binders of bioactive helical peptides. *Nature* 626:435–42
96. Walsh G, Walsh E. 2022. Biopharmaceutical benchmarks 2022. *Nat. Biotechnol.* 40:1722–60
97. Waltz E. 2020. A biotech insect repellent, safe enough to eat. *Nat. Biotechnol.* 38:1368–69
98. Wang F, Lv X, Xie W, Zhou P, Zhu Y, et al. 2017. Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve isoprene production in *Saccharomyces cerevisiae*. *Metab. Eng.* 39:257–66

99. Wang G, Björk SM, Huang M, Liu Q, Campbell K, et al. 2019. RNAi expression tuning, microfluidic screening, and genome recombining for improved protein production in *Saccharomyces cerevisiae*. *PNAS* 116:9324–32
100. Wang S, Zhan C, Nie S, Tian D, Lu J, et al. 2023. Enzyme and metabolic engineering strategies for biosynthesis of  $\alpha$ -farnesene in *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* 71:12452–61
101. Weber E, Engler C, Gruetzner R, Werner S, Marillonnet S. 2011. A modular cloning system for standardized assembly of multigene constructs. *PLOS ONE* 6:e16765
102. Wong BG, Mancuso CP, Kiriakov S, Bashor CJ, Khalil AS. 2018. Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. *Nat. Biotechnol.* 36:614–23
103. Wu R, Chen D, Cao S, Lu Z, Huang J, et al. 2020. Enhanced ethanol production from sugarcane molasses by industrially engineered *Saccharomyces cerevisiae* via replacement of the *PHO4* gene. *RSC Adv.* 10:2267–76
104. Xu Y, Wang X, Zhang C, Zhou X, Xu X, et al. 2022. De novo biosynthesis of rubusoside and rebaudiosides in engineered yeasts. *Nat. Commun.* 13:3040
105. Yu T, Zhou YJ, Huang M, Liu Q, Pereira R, et al. 2018. Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis. *Cell* 174:1549–58.e14
106. Yue Q, Meng J, Qiu Y, Yin M, Zhang L, et al. 2023. A polycistronic system for multiplexed and precalibrated expression of multigene pathways in fungi. *Nat. Commun.* 14:4267
107. Zhang J, Hansen LG, Gudich O, Viehrig K, Lassen LMM, et al. 2022. A microbial supply chain for production of the anti-cancer drug vinblastine. *Nature* 609:341–47
108. Zhang Y, Su M, Chen Y, Wang Z, Nielsen J, Liu Z. 2023. Engineering yeast mitochondrial metabolism for 3-hydroxypropionate production. *Biotechnol. Biofuels Bioprod.* 16:64
109. Zhang Y, Wang J, Wang Z, Zhang Y, Shi S, et al. 2019. A gRNA-tRNA array CRISPR-Cas9 based rapid multiplexed genome editing in *Saccharomyces cerevisiae*. *Nat. Commun.* 10:1053
110. Zhao M, Zhao Y, Yao M, Iqbal H, Hu Q, et al. 2020. Pathway engineering in yeast for synthesizing the complex polyketide bikaverin. *Nat. Commun.* 11:6197
111. Zhong W, Li H, Wang Y. 2023. Design and construction of artificial biological systems for one-carbon utilization. *BioDesign Res.* 5:0021
112. Zhou YJ, Buijs NA, Zhu Z, Qin J, Siewers V, Nielsen J. 2016. Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nat. Commun.* 7:11709
113. Zhu J, Alegre-Requena JV, Cherry P, Curtis D, Harvey BG, et al. 2023. Sooting tendencies of terpenes and hydrogenated terpenes as sustainable transportation biofuels. *Proc. Combust. Inst.* 39:877–87
114. Zhuang Y, Yang G-Y, Chen X, Liu Q, Zhang X, et al. 2017. Biosynthesis of plant-derived ginsenoside Rh2 in yeast via repurposing a key promiscuous microbial enzyme. *Metab. Eng.* 42:25–32