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Innovative tools and strategies for optimizing yeast cell factories

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Metabolic engineering; genome editing; cis/trans regulators; metabolic fluxes; subcellular compartmentation; adaptive laboratory evolution.

Abstract (100 - 120 words) [119 words]

Metabolic engineering aims to develop efficient microbial cell factories that can produce a wide variety of valuable compounds, ideally at the highest yield and from various feedstocks. In this review, we summarize recent developments in metabolic engineering approaches to tailor different yeast cell factories. In particular, we highlight the most timely and cutting-edge molecular tools and strategies for biosynthetic pathways optimization (including genome editing tools), combinatorial transcriptional and post-transcriptional engineering (*cis/trans* regulators), dynamic control of metabolic fluxes (e.g. rewiring of primary metabolism), and spatial reconfiguration of metabolic pathways. Finally, we discuss some challenges and perspectives for the adaptive laboratory evolution of yeast to advance metabolic engineering in microbial cell factories.

Glossary (450 words, strongly recommended) [403words]

adaptive laboratory evolution (ALE): integrated approach relying on the capacity of microorganisms to evolve in response to specific cultivation conditions, under selection pressure, and along an extended period (hundreds to thousands of generations), to obtain improved strains (growth, tolerance, titer, etc).

carbon source response elements (CSRE): short promoter sequences, identified as activating motifs of yeast gluconeogenic genes, and responsive to ethanol.

cell surface engineering (CSE): recombinant protein expression and incorporation into the yeast cell wall, to generate a whole-cell catalyst capable to accommodate different enzymatic reactions directly on the extracellular interface.

cellulosome: multi-enzyme complexes associated with the cell surface of cellulolytic microorganisms, mediating cell attachment to insoluble substrates for degradation into soluble products.

“Design, Build, Test, Learn” (DBTL) cycle: an integrated iterative approach for metabolic engineering of high-performance microbial biocatalysts and improvement of the commercially relevant metrics of titer, rate, and yield.

genome editing: genetic modification using genome editing tools that result in DNA deletion, integration or substitution within a genome.

global fitness: physiological state of living cells at a given time.

metabolic biosensors: biomolecules able to detect/respond to specific metabolites within metabolic pathways.

metabolic engineering (ME): an optimization process of cellular activity to enhance/adjust the production of desired compounds.

metabolic flux: a movement of metabolites through metabolic pathways over time, which characterizes enzymatic activity.

metabolite responsive allosteric transcription factors (aTFs or MRTFs): activator/repressor type transcription factors, which are activated through conformational changes upon the direct interaction with metabolites via specific inactive binding sites.

metabolons: molecular complexes that are dynamically formed within a metabolic pathway, including enzymes, cellular structures and annex proteins.

natural products (NPs): chemical compounds found in nature, including functional specialized metabolites produced by microorganisms (mostly bacteria and molds…), mushrooms, marine animals, algae, or land plants, often used in human pharmacopeia.

spatial reconfiguration: the modification of protein subcellular localization according to the employed strategies via truncation/substitution/addition of targeting sequences.

synthetic biology (SB): a recently emerged multidisciplinary research area that focuses on the *de novo* engineering/(re)design of biological components with user-defined features.

titer, rate, and yield (TRY): refers to production metrics of product concentration, the time required to generate it, and the final amount.

yeast cell factories (YCFs): an approach in bio-engineering, which relies on metabolic engineering and employs yeast cells as a production unit.

Highlights (900 characters, including spaces, required) [881 char.]

- Bio-production of many medicinal natural products suffering from shortage or low availability is the best option to ensure a stable supply to the pharmaceutical industry.
- Among all the microorganisms, yeasts (including *Saccharomyces cerevisiae* and other non-conventional strains) constitute highly valuable platforms for industrial bio-production of natural products.
- Progress in metabolic engineering and synthetic biology enabled the development of yeast cell factories capable of producing natural products efficiently.
- Implementation of yeast cell factories relies on molecular tools and strategies for the optimization of biosynthetic pathways, dynamic control and spatial (re)configuration of metabolic fluxes *in vivo*.
- Several of these tools and strategies still have limitations, however, constant effort in the field is made to overcome it and to optimize yeast cell factories.

Outstanding Questions Box (2000 characters, including spaces, required) [753 char.]

- How can we accelerate the establishment of yeast cell factories?
- How can we rationally diversify the palette of target compounds for bio-production?

- How can we develop efficient tools for subtle functionalizations (chlorination, fluorination…) of target compounds in yeast?
- How can we find the best yeast chassis for each application?
- How can we efficiently evaluate/predict the output of different metabolic engineering modifications applied during the development of yeast cell factories?
- How can we further combine artificial intelligence (design; learn) and automation (build; test) to improve metabolic engineering approaches?
- How can we accelerate the scale-up for the bio-production of target compounds by yeast cell factories at an industrial scale?

Text body [4522/4000]

Towards the ideal yeast cell factory: from rational design to adaptive laboratory evolution

Medicinal **natural products (NPs)** (see Glossary) suffer from recurrent shortages, mainly due to the overexploitation of their sources [1-4]. While the well-known and long-established uses of synthetic chemistry (SC) allow producing many NPs and their derivatives, at large scales, some compounds such as plant-derived NPs (monoterpene indole alkaloids (MIAs); benzylisoquinoline alkaloids (BIAs)…) and mammal derived NPs (hormones; neurotransmitters…) are too complex molecules (asymmetric carbons, long multi-step biosynthetic pathway…) to be synthesized by SC alone at an industrial scale [4–9]. Although recent progress made in SC allows nowadays to produce very complex NPs such as strictosidine [8], chemical synthesis is not always the most suitable approach in terms of ecological impact and cost efficiency, therefore, alternative strategies have been considered to produce these NPs in greater quantities, such as using recombinant microorganisms (Fig. 1, Key Figure). Microorganisms such as yeast have evolved to maintain constant metabolic homeostasis regardless of rapidly changing environmental conditions, so intensively rewiring their metabolism is essential for bioproduction of NPs with high **titer, rate, and yield (TRY)** at scale [6, 7]. Several examples of successful heterogeneous biosynthesis of mammal/plant NPs can be cited (progesterone [9], hydrocortisone [10, 11]/ artemisinic acid [12–14], resveratrol [15], strictosidine [3, 16], (S)-reticuline [17]), only few, nevertheless, reached an industrial-scale production. For instance, the commercialization of artemisinic acid biosynthesis (a precursor of antimalarial artemisinin), produced at high levels by *Saccharomyces cerevisiae*, did not persist due to poor market demand [14]. In contrast, the major mammal anti-inflammatory hormone hydrocortisone is synthesized by *S. cerevisiae* [10, 11], which is still the main hydrocortisone producer. However, many NPs, particularly highly valuable plant pharmaceuticals (such as anticancer vinblastine and vincristine, or opioids) are still not able to be produced by recombinant

151 microorganisms efficiently due to multiple limitations [1-7], which will be discussed in this review.
152 In the recent years, **metabolic engineering (ME),** which aims to rewire cellular metabolism, has
153 utterly empowered and accelerated the development of **yeast cell factories (YCFs)** by
154 enhancing the TRY of the bio-produced target compounds, broadening the spectrum of these
155 compounds (e.g., taxol, opioids), increasing the range of substrates (e.g., xylose, arabinose),
156 and enhancing strain physiological properties (e.g., global fitness, stress tolerance) [3–7]. In
157 particular, baker's yeast (*Saccharomyces cerevisiae*), as well as some non-conventional yeasts
158 (*Pichia pastoris*, *Yarrowia lipolytica;* with specific features of interest, such as high secretion
159 ability or lipophilic compounds accumulation [18, 19]), generally regarded as safe (GRAS) and
160 robust microorganisms, have their genome entirely known and easy to manipulate. In recent
161 decades, remarkable progress has been made in the field of molecular and cell biology of yeast,
162 due to the rapid development of genome sequencing, cell engineering and **synthetic biology**
163 **(SB)** [4–7]. However, many limiting factors remain that make genome manipulation and the
164 construction of YCFs inefficient and time-consuming [6, 7]. Indeed, creating YCFs commonly
165 implies the integration of complex biosynthetic pathways, which requires the high-fidelity
166 assembly of long DNA fragments and sophisticated **genome editing** tools [18, 20, 21]. Novel
167 strategies and approaches are continuously developing to facilitate multiplex marker-free
168 genomic integration, DNA assembly and transformation efficiency [5–7, 20, 22]. Besides,
169 integrating heterologous pathways in yeast generally involves fine-tuning gene expression,
170 which has been limited by the availability of characterized native gene regulatory elements,
171 because they are lengthy and unable to cover the required range of the expression strength [23–
172 25]. Multiple approaches aiming at optimizing and extending transcription regulation [26, 27] are
173 still constantly evolving to further broaden the assortment of regulatory elements and overcome
174 their limitations. Moreover, the overexpression of heterologous biosynthetic pathways is
175 frequently responsible for massive stress affecting global cell fitness, which includes the
176 accumulation of toxic intermediates, carbon source competition, and a loss of reducing power

(i.e. oxidative stress, and/or unbalanced/competitive use of cofactors) [6, 7, 22]. In particular, developing an industrial bioprocess based on YCFs compatible with commercial purposes, with a maximal product TRY and optimal metabolic fluxes of the multiple integrated genes, demands precise control and balance and remains a key goal for already-established recombinant strains [28, 29].

Key figure

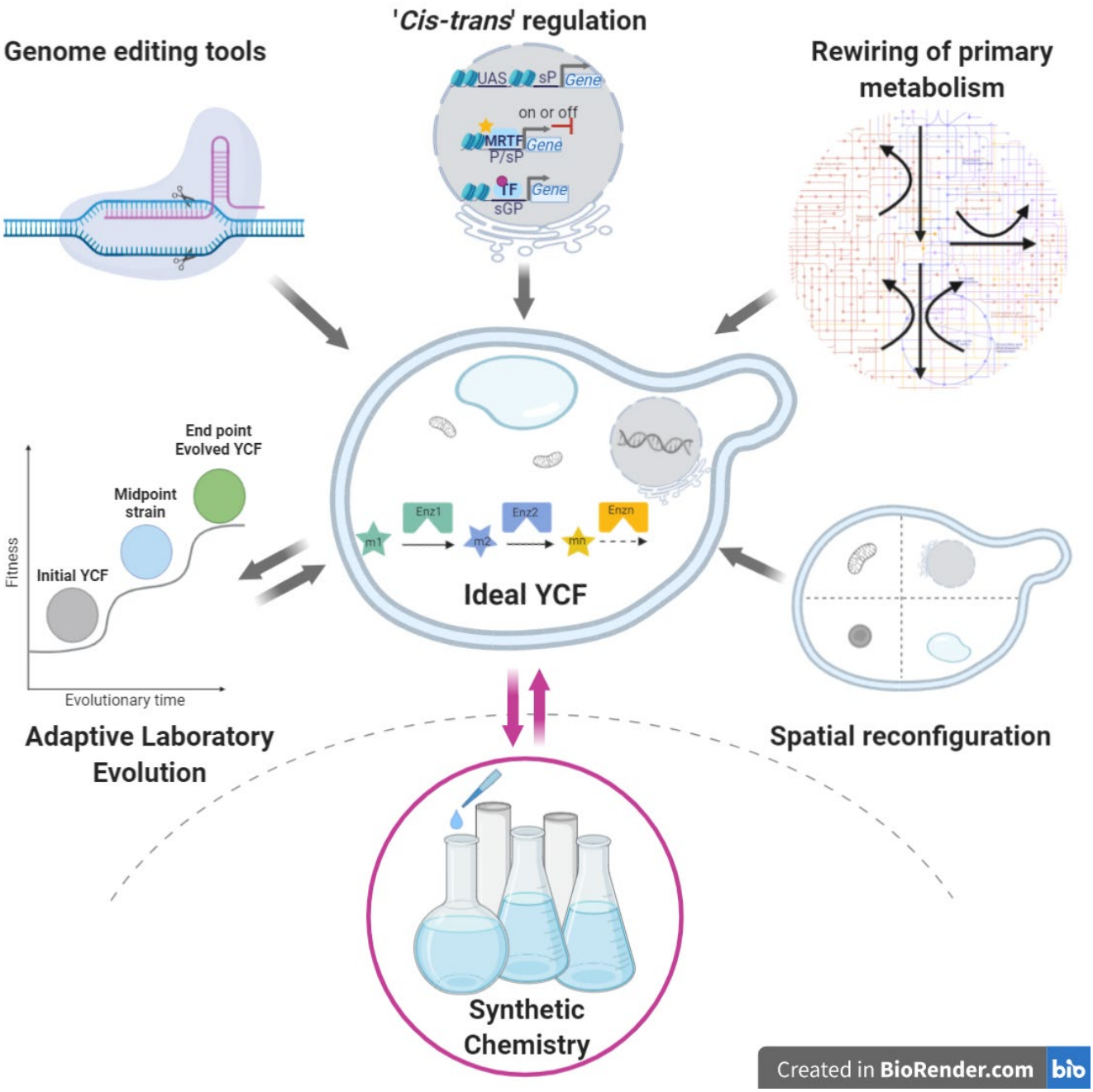

Figure 1, Key Figure: Comprehensive overview of cutting-edge molecular tools and strategies to implement the ideal Yeast Cell Factory (YCF). This figure illustrates various synthetic biology tools that are employed for the YCF construction and optimization (grey arrows), such as advanced genome editing tools, transcriptional *("cis")* and post-transcriptional *("trans")* regulation, rewiring of yeast primary metabolism and adaptive laboratory evolution (ALE), which will be discussed in this review. However, heterologous production also presents several limitations/challenges, which, in tandem with synthetic chemistry (purple circle and arrows), potentially can be resolved (e.g. substrate chemical synthesis, product modification), and represent promising prospects.

Furthermore, enzyme and substrate differential subcellular localization [30–32], enzymatic promiscuity, and pathway lateral branches generally decrease substrate availability and metabolic fluxes by hijacking biosynthetic intermediates [33, 34]. As such, formal strategies are also required to redesign metabolic pathways in yeast. This work is a comprehensive review providing the reader with an integrative and clear view on the most recent and innovative molecular tools and approaches dedicated to the implementation and improvement of YCFs for the bioproduction of valuable molecules. It will especially emphasize specific challenges associated with already existing methods, and discuss successful examples that highlight the latest tools and advances, and future perspectives.

Genome editing tools

In yeast genome editing, homologous recombination and cre/lox-mediated integration were replaced by the CRISPR/Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats CRISPR, and CRISPR-associated protein 9 - nuclease Cas9 from *Streptococcus pyogenes*), which is still continuously improving to facilitate and accelerate DNA assembly and genome manipulation [18, 21]. Particularly laborious was the assembly of large template DNA (multiple expression cassettes), which is now generally realized *in vivo* via overlapping single-stranded oligonucleotides and homologous recombination (HR), the technique described as DNA assembler [35]. It bypasses multistep and multi plasmid cloning and was successfully employed in an assembly of up to eight-gene biosynthetic pathways in *S. cerevisiae* with the efficiency being inversely correlated to the number of genes [35]. Another limiting factor

consisted of multiplex genomic integration, which requires multiple gRNAs. Recently, several novel approaches were developed, aimed to perform multisite targeting and exploiting the simultaneous expression of numerous gRNA using Cas9-expressing yeast strains or all-in-one plasmids (**Fig. 2A**). For instance, in the CasEMBLR method (Cas9-facilitated multi loci integration of assembled DNA parts into *S. cerevisiae* chromosomes, in combination with DNA assembler techniques), DNA fragments are amplified by PCR to contain homologous overhangs for further *in vivo* assembly, and marker-free integration was reported to be successful into up to five sites with 50-100% efficiency, which implies both gRNAs-expressing plasmid and Cas9-expressing yeast strain [36, 37]. All-in-one plasmids, such as gRNAs-Cas9 co-expressing vectors with an inducible promoter for Cas9 (also available in constitutive version), enable dissociating cloning from genome editing by yeast pre-transformation with all CRISPR/Cas9 components prior to the introduction of template DNA [38]. Time-effectively, the all-in-one plasmids contain a universal gRNA expression cassette, and the introduction of an appropriate gRNA targeting sequence upon plasmid recircularization takes place *in vivo* in yeast via HR [38]. However, the conventional expression and delivery of multiple gRNAs are delicate, either an individual expression cassette is required for each gRNA, or a common transcript is generated and further cleaved via various strategies into individual gRNAs [39]. Both approaches are limited by the number of gRNAs expressed in the system and the processing efficiency. Recently, alternative strategies were reported. In the GTR-CRISPR (**Fig. 2B**) (tandem gRNA-tRNA array for CRISPR/Cas9) the use of a gRNA-tRNA array within the Cas9-expressing plasmid, and exploiting the endogenous tRNA-processing system, which precisely cleaves the precursor of tRNA and enables the release of single gRNAs from the common gRNA-tRNA transcript, resulted in the simultaneous disruption of 8 genes in *S. cerevisiae* with 87% efficiency [40]. Moreover, Lightning GTR-CRISPR, an *E.coli*-free Golden Gate cloning system [41]-compatible version can be employed (the Golden Gate reaction is directly transformed into yeast), which was shown to result in 96% and 60% efficiency of correct editing, illustrated through the

disruption of four and six genes, respectively [40]. Alternatively, a plasmid-free gRNA delivery method gRNA- Transient Expression System (TES) was described, where gRNA-encoding PCR fragments, composed of the promoter, guiding sequence and gRNA scaffold (two gRNAs are required to cleave both sides of the targeted region), are transformed together with template DNA into Cas9-expressing *S. cerevisiae* strain, which resulted in the substitution of different sized regions of *S. cerevisiae* chromosome 4 by template DNA with 67-100% efficiency [42].

In parallel, the CRISPR/Cas9 system has been explored to enhance the efficiency of its components (**Fig. 2B**). In this context Cpf1 (CRISPR from Prevotella and Francisella 1), a family of class 2/type V CRISPR bacterial endonucleases was found to display several advantages compared to Cas9. For instance, Cpf1 generates sticky ends instead of blunt ends upon DNA cleavage [43], which potentially facilitates template DNA integration. Moreover, Cfp1 possesses both RNAase and DNAse activities and does not need RNAse III, which results in the requirement of only crispr RNA (crRNA) instead of a longer complex of *trans*-activating crispr RNA (tracrRNA) and crRNA [43, 44]. Thus, *S. cerevisiae* strain producing patchoulol was generated using self-cloning Cpf1-crRNA co-expressing plasmid, where singleplex and triplex genomic integrations of *in vivo* assembled template DNA were achieved with 80% and 32% of efficiency, respectively [45].

In addition, dCas9-mediated Target-AID (Activation Induced cytidine Deaminase from vertebrate), a synthetic hybrid complex that performs highly efficient C to G and C to T mutations, was successfully used in high throughput loss of function screens [46], which is an appealing application to screen for yeast *de novo* features. On the other hand, the σ sequences, which are a family of repetitive DNA sequences (at least 100 copies) in the *S. cerevisiae* genome [47], were used together with the CRISPR/Cas9 system in a novel method of genome shuffling [48]. Cas9, guided by the gRNA targeting the σ sequences, cleaves DNA at multiple sites, thus promoting endogenous DNA repair and mutagenesis [49], which can lead to the improvement of yeast characteristics when cultivated under specific conditions (e.g. thermotolerant *S.*

cerevisiae obtained under high-temperature conditions [48]). These approaches demonstrate the application of the CRISPR/Cas9 system beyond gene integration, notably useful in **adaptive laboratory evolution (ALE)**, which will be presented in the last section of this review.

Transcriptional ("cis") and post-transcriptional ("trans") regulator toolboxes

Alteration and regulation of gene expression at the transcriptional level via promoter and terminator elements have been unceasingly considered as a powerful approach. Previously characterized long native elements were initially employed (*S. cerevisiae* p*TEF1*, p*GAL1-10*, t*CYC1*, t*ADH1,* etc) [23–25], which, however, pointed out an extensive necessity of development of novel synthetic units, more compact and functionally diversified. Indeed, the integration of complex multigenic heterologous pathways in yeast mobilizes a promoter and a terminator for each gene, which considerably extends template DNA length, increases the risk of self-recombination, as well as generally demands optimization of gene expression. To address these issues, several regulatory sequence libraries were recently constructed and investigated (**Fig. 3A**). For instance, a library of short 69-bp semi-synthetic promoters, covering an 8.0-fold expression range, was generated from the determined minimal length of *S. cerevisiae* native p*TEF1* [50] **(Fig. 3A1)**. Moreover, the use of upstream activating sequences (UAS) expanded the library to reach a 20-fold expression range and a maximal length of 130 bp. Importantly, some of the p*TEF1* variants demonstrated expression strength comparable to native p*TEF1* and p*PGK1*. Another library of *Y. lipolytica* semi-synthetic promoters was generated, also based on the promoter core region between the TATA-box and the transcriptional starting site (TSS) upstream the 5' untranslated region (5'UTR) sequence [51] **(Fig. 3A2)**. Artificial sequences of 30 bp in length were designed (various combinations of T-rich and G/C-rich fragments) and substituted in *Y. lipolytica* strong native p*EXP1* and p*GDP* to assess expression levels of crtY enzyme and, consequently, conversion of lycopene to β-carotene. The results demonstrated a

population of novel *Y. lipolytica* promoters showing an up to a 5.5-fold increase in lycopene conversion. On the other hand, the impact of 10-bp terminator linker 1 (the sequence between the efficiency element and the positioning element) on gene expression was investigated via synthetic terminator library in *S. cerevisiae* [52] **(Fig. 3A3)**. A 6.0-fold expression range was achieved and it was demonstrated that sequences with low GC content and enriched in T were conferring higher levels of expression.

In addition to constitutive regulation of gene expression, **metabolic biosensors** play a central role in metabolic rewiring and the optimization of productivity in YCFs. For instance, **Metabolite responsive allosteric transcription factors (aTFs or MRTFs)** from bacteria interact with their corresponding metabolites prior to acting as repressors or activators of transcription [53]. To alter transcription, MRTFs bind promoter-specific sequences and, therefore, restrict or facilitate the access to RNA polymerase [53]. Thus, MRTFs enable dynamic control of heterologous gene expression and, consequently, regulate metabolic activity. However, the engineering of MRTFs in eukaryotic cells remains more than challenging. Recently, a novel strategy has been reported and aimed to generate user-defined biosensors via evolution-guided toggled selection (directed biosensor evolution and library construction, followed by the selection of mutants via alternating conditions according to the set criteria), based on the mutagenesis of aTF effector binding domain (EBD) and Fluorescence-Activated Cell Sorting (FACS)-mediated selection [54] (**Fig. 3B1**). Thus, a variety of *de novo* features were identified, such as inversion of function and change of specificity, as well as modification of dynamic and operational range. Another approach focused on the optimization of biosensor reporter promoters in a binding site-dependent manner [55] (**Fig. 3B2**). The constructed synthetic promoter libraries (from native p*TEF1* and truncated p*CYC1*) covered all the possible positions for introducing aTFs binding site within promoter core region, which resulted in the identification of function-related positions for most of the screened repressor or activator type aTFs [55]. The complementary aspect of gene expression regulation via promoters is related to the carbon source of yeast metabolism.

Given that multiple commonly used native promoters are associated with glycolytic genes and are less effective under glucose starvation [25], novel promoters, activated upon diauxic shift, are required to maintain constitutive gene expression (or to disassociate growth from production if required). Recently, **carbon source response elements (CSRE)** of gluconeogenic promoters were introduced upstream UAS of *S. cerevisiae* p*TDH3*, substituting the binding site of glycolytic genes transcriptional activator Gcr1 and surrounding neutral sequence [56] (**Fig. 3C**). This approach demonstrated strong induction of reporter Yellow Fluorescent Protein (YFP) in the low-glucose medium instead of a glucose-rich medium, which was validated in a vanillin-β-glucoside-producing yeast strain. Indeed, when glycolytic promoters were employed, the production of vanillin-β-glucoside took place mostly during the ethanol phase leading to the accumulation of the cytotoxic intermediate protocatechuic acid (PCA). The use of generated gluconeogenic promoters to control the expression of enzymes converting PCA to vanillin-β-glucoside showed an enhanced yeast growth, lower accumulation of PCA, and conserved vanillin-β-glucoside bioconversion [56]. Taken together, these advances are broadening the collection of available gene regulation elements and deliver compelling guidance to combinatorial approaches for metabolic rewiring in YCF.

Rewiring of primary metabolism strategies

The strategies to enhance TRY imply the optimization of host primary metabolism, which includes the modification of native metabolic fluxes and coupling/decoupling growth with production. Sufficient availability of the NADPH cofactor, required by numerous enzymes, in some cases (excluding, for instance, hydrocortisone biosynthesis [10]) stands as a limiting factor (e.g. *S. cerevisiae* 3-hydroxy-3-methylglutaryl-CoA reductase Hmg1, *S. cerevisiae* squalene synthase Erg9 etc) [57, 58], given that glucose is dispatched in both glycolysis and pentose phosphate pathway (PPP) upon consumption [59]. Several successful approaches have been

implemented to enhance NADPH (re)generation (**Fig. 4A**) and, therefore, heterologous/homologous production. For instance, a number of *S. cerevisiae* genes involved in NADPH synthesis were deleted or overexpressed to assess the effect on NADPH concentration in the cytosol and heterologous protopanaxadiol (PPD) production from endogenous squalene [57]. In the best performing PPD-producing *S. cerevisiae* strain, the deletion of NADH-generating *ALD2* and overexpression of NADPH-generating *ALD6* aldehyde dehydrogenases-encoding genes, involved in ethanol metabolism, resulted in a 1.3-fold increase of NADPH cytosolic concentration and a 4.5-fold increase in PPD production [57]. Likewise, the positive effect of the overexpression of the Ald6-encoding gene was demonstrated in recombinant noscapine-producing *S. cerevisiae* [60]. The overexpression of *S. cerevisiae* full-length mitochondrial or truncated cytoplasmic NADH kinase Pos5, which catalyzes NADPH-generating reactions, led to the 9.0-fold and 7.0-fold enhancement of homologous squalene production respectively [58]. Similarly, the overexpression of truncated cytoplasmic Pos5 in antibody fragment-expressing *P. pastoris* was shown to significantly increase the production of recombinant protein [61].

Another important cofactor in cellular metabolism is *S*-adenosylmethionine (SAM), which is a donor of the methyl group upon methylation catalyzed by SAM-dependent methyltransferases (Mtases) [62]. Methylation, being required by a wide range of cellular processes, is used in biotechnological approaches for the heterologous production of valuable methylated compounds [63]. Engineering of SAM-dependent Mtases has been challenging, mostly due to the lack of efficiency and consistency. Recently, methylation was coupled to growth by combining enhanced Mtase activity and cysteine biosynthesis [64] (**Fig. 4B**), which was achieved via Mtase adaptive laboratory evolution *in vivo* and deletion of several *S. cerevisiae* genes. On the other hand, certain enzymes require specific chemical elements for their formation and activity such as the bacterial xylonate dehydratase (XylD, involved in xylose metabolism) that contains iron-sulfur (Fe-S) cluster [65]. Although *S. cerevisiae* Fe metabolism was not reported to be limiting

for bovine Fe-S adrenodoxin and hydrocortisone biosynthesis [10], the folding and the activity of several bacterial Fe-S enzymes were reported to be insufficient in yeast [66–68], still being a key concern in the functional expression of bacterial Fe-S proteins. Recently, Fe metabolism of yeast cytosolic Fe-S cluster machinery was modified to enhance Fe uptake, XylD generation, and 1,2,4-butanetriol production [65] (**Fig. 4C**). In the best performing recombinant *S. cerevisiae* strain, the overexpression of truncated Tyw1 protein (tTyw1), unable to bind and sequestrate Fe-S clusters, and the deletion of one of the components of negative regulation of Fe uptake, resulted in 6.0-fold and 1.4-fold higher XylD activity and 1,2,4-butanetriol production, respectively [65].

Spatial reconfiguration strategies

The development of valuable compound-producing YCFs usually involves complex enzymatic pathways, requiring specific physicochemical conditions, substrates and cofactors, potentially affected by adverse side reactions or toxic intermediates [7, 20]. To meet these challenges, several approaches based on the spatial rearrangement of the desired pathway(s) have emerged, from the metabolic engineering of organelles (MEO) to the construction of artificial **metabolons** [30–32]. Recently, successful artificial compartmentalization of the triterpene biosynthetic pathway in the peroxisome of *S. cerevisiae* allowed producing a high concentration of squalene (**Fig. 5A**) revealing this organelle as a promising site for the biosynthesis and storage of terpene compounds [69]. In particular, the rapid and highly efficient protein import machinery of peroxisomes, along with their high plasticity (number and size dynamically adjusted according to the physiological state of the cells), combined to a channeling effect insured by the close vicinity of overexpressed heterologous biosynthetic enzymes, make this subcellular compartment an ideal target for improvement of YCFs.

Previously, the successful bioproduction of hydrocortisone in yeast elegantly showed the flexibility of yeast in accommodating the relocalization of membrane-bound enzymes to a

different subcellular compartment, without affecting the final titer of hydrocortisone produced [10, 11]. More recently, the successful artificial compartmentalization of isoprenoid biosynthesis into the mitochondria was achieved (**Fig. 5B**), with an enhanced supply of acetyl-CoA and tricarboxylic acid cycle intermediates [70]. However, as mitochondrion is gathering crowds of essential proteins involved in respiration, the compartmentalization of heterologous biosynthetic pathways in this organelle might be, in some cases, responsible for metabolic stresses. In MEO, important physiological parameters such as the size and the biogenesis of organelles can increase the physical space available for enzyme encapsulation and storage of metabolites [71–74]. For example, increasing the size of lipid droplets, by modulating triacylglycerol metabolism, allowed a 1.25-fold increase in lycopene bio-production in a recombinant strain of *S. cerevisiae*, correlated to the improved storage capacity of hydrophilic lycopene [74]. Similarly, the overexpression of a key ER size regulatory factor gene, *INO2*, allowed a significant increase of the surface of the ER [73], associated with a drastic augmentation of production of P450s [71-73], and lead to an 8-fold increase in PPD bio-production, along with a 7.1-fold increase of protein secretion, emphasizing the crucial role of ER in protein synthesis and folding, to circumvent potential metabolic constraints [73, 75].

In parallel to MEO, another approach has emerged allowing the display of enzymes directly at the surface of the yeast cells (**Fig. 5C**) [76]. The **cell surface engineering (CSE)** approach enables the generation of the whole cell catalysts to achieve the hydrolysis of numerous substrates, including lignocellulosic biomass, consecutively converted by fermentation into a variety of valuable compounds such as ethanol and xylitol [31, 76–78]. In brief, CSE consists of targeting heterologous enzymes to the cell wall, through the secretion pathway, to expose their catalytic sites toward the extracellular environment. The enzymes can be displayed either individually or gathered in association with a protein-based scaffold to form the **cellulosome**, a multi-enzymatic complex structure capable of accommodating up to 63 enzymes for the largest one reported to date [31, 78]. One of the main challenges in CSE resides in controlling the

efficiency of the display, as well as the anchorage position of the target protein within the cell wall. In the case of the bio-production of xylitol from lignocellulose by CSE, the uptake of xylose across the membrane of the cells constitutes a critical point, as the bioconversion reaction requires NADH and cannot be achieved at the surface of the cells [77, 79]. Interestingly, the co-expression of a homologous maltose transporter (*Sc*Mal11) along with a beta-glucosidase (*Aa*Bgl) degrading cello-oligosaccharides (e.g. cellobiose) on the cell surface of *S. cerevisiae* allowed to enhancing xylitol production from the co-utilization of cellobiose/xylose contained in lignocellulose [80].

To prevent the accumulation of toxic/unstable intermediates as well as the loss of the desired intermediates, another interesting option has emerged based on the spatial (re)arrangement of enzymes into artificial metabolon, ensuring substrate channeling for a continuous metabolic flux within the YCF [32, 81]. Two main approaches enable constructing such artificial metabolons: one consisting of a direct fusion of the enzymes to each other (protein linker), and one relying on the interaction of enzymes with protein (or nucleic acid)-based scaffolds (**Fig. 5D**). While protein fusion is considered as the easiest way to enhance substrate channeling, this approach may alter the structure of the enzymes and is always restricted to a very limited (two to three) number of enzymes. Nevertheless, the over-expression of an engineered tridomain enzyme (CrtB, CrtI, CrtY) harboring the full β-carotene biosynthetic pathway lead to an improved bio-production of the pigment in *S. cerevisiae* highlighting the potential of this approach for the improvement of YCFs [81]. On another hand, artificial metabolon using protein-based scaffold technologies allows the gathering of several enzymes in close vicinity through affinity binding, leading to significantly improved xylose utilization and resveratrol bio-production in *S. cerevisiae* and to decrease the accumulation of by-product xylitol [82].

Adaptive laboratory evolution strategies

Besides pathway engineering and metabolic flux rewiring approaches, the development of robust YCFs is crucial for industrial application purposes, due to harsh culture conditions and frequent limiting toxicity of intermediates and products [83, 84]. Adaptive laboratory evolution (ALE) relies on yeast adaptation capacity allowed by multiple DNA recombination events and high genomic plasticity, utterly difficult to achieve through the rational engineering approach alone (**Fig. 6A**) [83]. For instance, ALE led to improved xylose utilization ability in *S. cerevisiae* along with an enhanced isobutanol bioproduction due to point mutations in the *CCR4* and *TIF1* genes, and fine-tuning of gene expression in the evolved strain (**Fig. 6B**) [85]. Associated with high throughput screening methodologies [84, 86], ALE constitutes a powerful approach allowing speeding up the conception of highly efficient recombinant YCFs (**Fig. 6C-E**). In particular, ALE allows overcoming numerous physiological limitations (i.e. thermotolerance, osmotic stress, low pH, toxicity, etc) [48, 84–92] as well as to complement the rational engineering approaches presented in the above sections, and providing new targets for the next round of rational design [64, 83–86, 92–102]. Noteworthily, the use of CRISPR/Cas9 system in combination with ALE approach enabled the generation of evolved YCF with improved thermotolerance, as mentioned in the "*Genome editing*" section of this review [48]. Other stricking studies, such as growth-coupling strategies associated to ALE, can be cited [64, 103]. In particular, when the production of the essential amino acid cysteine was tied to the activity of methyltransferases, ALE was used to select for both *E. coli* and *S. cerevisiae* strains with mutations leading to 2-fold increases in heterologous methyltransferase activity [64]. In this study, adaptive mutations were forced to preferentially target the methyltransferase activity bottleneck which was limiting for growth-rate. This approach allowed to significantly improve activity of both N- and O-type methyltransferases, as illustrated in the "*Rewiring of primary metabolism*" section of this review.

In addition, ALE virtually allows to improve the **"Design, Build, Test, Learn" (DBTL) cycle** classically used in ME to generate YCFs (**Fig. 6E**), when performed in the continuity of the "Build" step for rescuing/optimizing a strain with decreased fitness [83]. Nevertheless, given that

that evolution of the strains often drives the population towards a low-production high-fitness phenotype, which constitutes an important limitation of ALE, an extensive effort is still required to overcome this problem [83–85, 103, 104]. In that sense, combining ALE with multi-omic technologies is emerging as the most promising and efficient approach in YCFs engineering [48, 64, 84, 86, 95, 102, 104, 105].

Concluding remarks: Future Challenges and Directions

Using synthetic biology to implement YCFs is a promising approach to produce very complex compounds (e.g. vinblastine, vincristine…) almost impossible to obtain by SC alone (i.e. asymmetric carbons). However, although several molecular tools (e.g. halogenases) have been characterized in marine bacteria and plants, reports on bioproduction of halogenated NPs in yeast is scarce [106-108]. This kind of subtle modification (chlorination, fluorination…) remains therefore as a future challenge for YCFs engineering to generate new-to-nature compounds with high pharmaceutical interest (see Outstanding Questions) [1-3, 106-109]. Recently, an indirect way to obtain such compounds was reported, consisting in incorporating precursor derivatives (i.e. halogenated tyrosine) to produce S-reticuline derivatives [60]. Another recent study elegantly showed the bioproduction of halogenated oxo-(2-aminophenyl) and quinoline scaffolds in *S. cerevisiae*, by overexpressing regiospecific L-tryptophan halogenases [109]. Therefore SC is still indispensable to generate valuable fluorinated or chlorinated NPs derivatives, usually presenting much higher pharmacodynamic characteristics, and a larger demand in drug industries. Like for instance, the fluorinated derivative of hydrocortisone, namely dexamethasone, which is 40-times more potent than hydrocortisone and far more stable *in vivo*. In that sense, a strong complementarity between SB and SC exists, while SB is a highly sustainable and ecological way to produce platform compounds, SC can be employed in the compound downstream fine-tuning/modification and the upstream precursor-directed yeast-

503 mediated biosynthesis (bioconversion, [110]). Despite some technical limitations, yeast cells
504 represent a proficient eukaryotic tool, suitable for the bio-production of valuable and complex
505 molecules. Meanwhile, constant progress in SB has accelerated the industrial applications of
506 YCFs [2–7]. Besides model yeasts such as *S. cerevisiae*, several non-conventional chassis
507 present increasing interest in ME applications, due to their unique features [18, 19]. While SC
508 has made huge progress in the recent years, allowing nowadays to synthesize molecules as
509 complex as strictosidine [8], tailoring the ideal YCFs capable of producing high-scale NPs as
510 well as their derivatives (e.g. chlorination, fluorination…) is essential to establish next-generation
511 bio-foundries as cost- and time-efficient alternatives for sustainable bio-production at an
512 industrial scale. The coming decade is undoubtedly going to be full of exciting improvements in
513 the YCFs-based technology, and even allow us to see the final convergence of SC and SB,
514 allowing the production of a truly infinite palette of complex molecules.
515
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Figure Legends

Figure 1, Key Figure: Comprehensive overview of cutting-edge molecular tools and strategies to implement the ideal Yeast Cell Factory (YCF). This figure illustrates various synthetic biology tools that are employed for the YCF construction and optimization (grey arrows), such as advanced genome editing tools, transcriptional *("cis")* and post-transcriptional *("trans")* regulation, rewiring of yeast primary metabolism and adaptive laboratory evolution (ALE), which will be discussed in this review. However, heterologous production also presents several limitations/challenges, which, in tandem with synthetic chemistry (purple circle and arrows), potentially can be resolved (e.g. substrate chemical synthesis, product modification), and represent promising prospects.

Figure 2. Advanced genome editing tools in yeast (**A**) DNA assembly and CRISPR/Cas9-mediated yeast transformations. Cas9 is expressed in yeast (plasmid-encoded under constitutive or inducible promoter, according to user needs). This is achieved either in tandem with gRNA(s) [38], followed by the Cas9-gRNA(s)-expressing strain transformation with template DNA, or yeast pre-transformation with Cas9-expressing plasmid only, further followed by the transformation of Cas9-expressing strain with template DNA and gRNA(s) plasmid [36, 37]. A recent gRNA delivery method Transient Expression System (TES) was described, where the PCR fragments encoding the gRNAs (and containing the promoter, the guiding sequence and the gRNA scaffold) are transformed together with template DNA into Cas9-expressing yeast strain [42]. The template DNA (multiple expression cassettes, composed of promoter and terminator for each gene of interest) is assembled *in vivo* via overlapping oligonucleotides (here represented as colored lines at the extremities of expression cassettes, black lines represent the so-called Arms - the sequences homologous to the specific yeast genomic regions) and Homologous Recombination (HR) [36, 37]. The schematic example of a single expression cassette is shown. (**B**) Advances in CRISPR/Cas9 system. The GTR-CRISPR (tandem gRNA-tRNA array for CRISPR/Cas9) is based on the use of a gRNA-tRNA array encoded in a plasmid with Cas9, and the native tRNA-processing system (the precursor of tRNA is cleaved, which releases the individual gRNAs from the common transcript) [40]. Cpf1 (CRISPR from Prevotella and Francisella 1 endonuclease) demonstrates several advantages compared to Cas9 (generation of sticky ends, RNAse and DNAse activity, requirement of only crRNA) [44]. dCas9-mediated Target-AID (Activation Induced cytidine Deaminase from vertebrate) is a synthetic hybrid complex, which performs C to G and C to T mutations with high accuracy [46].

Figure 3. New tools for the regulation of gene expression in yeast. (**A**) Generation of synthetic gene regulatory element libraries. Promoter (P) core region between the TATA box and the transcriptional starting site (TSS) was mutated. 1) The determined minimal sequence of *S. cerevisiae* p*TEF1* core region was mutated. The variants, with or without UASs, were screened by measuring yECitrine (yECit, yellow-green fluorescent protein) fluorescence [50]. 2) The artificial sequences enriched in T and/or G/C were substituted in *Y. lipolytica* p*EXP1* and p*GDP* core regions. The screening - colony-color spectrum due to crtY activity (accumulation of carotene pigments) [51]. 3) In the *S. cerevisiae* terminator library terminator (T) linker 1 sequence between the efficiency element (EE) and the positioning element (PE) was randomized. The screens were done using Green Fluorescent Protein (GFP) fluorescence as a

reporter [52]. (**B**) Engineering of metabolite sensing and downstream regulation in *S. cerevisiae*. 1) The method enables the design of user-defined MRTFs by random mutagenesis of the MRTF effector binding domain (EBD) upstream DNA binding domain (DBD). The selection of MRTFs with *de novo* features is GFP Fluorescence-Activated Cell Sorting (FACS) - based. ON state - dark green (presence of inducer), OFF state - light green (control medium). The dashed line rectangle - variant selection, the purple background - control strain not expressing MRTF. The criteria for selection vary according to user needs [54]. 2) Activator- or repressor-type MRTF binding site (BS) were inserted in front of every nucleotide of native p*TEF1* or truncated p*CYC1* core regions to generate MRTF operator library [55]. (**C**) Construction of synthetic gluconeogenic promoters in yeast [56]. Native promoters were reshaped from glycolytic to gluconeogenic via substitution of the glycolytic genes transcriptional activator Gcr1 BS by carbon source response elements (CSRE) of native gluconeogenic promoters. sP - synthetic promoter, purple and black circles/curves - ethanol and glucose, respectively. Overlapping of curves - diauxic shift.

Figure 4. Engineering of primary metabolism in yeast. (**A**) Redox engineering. PPP stands for the pentose phosphate pathway. The best performing strains are illustrated. Green color indicates overexpression while velvet color - deletion. *ALD6* was overexpressed [57, 60] and *ALD2* deleted [57]. Full-length *POS5*/truncated *tPOS5* were overexpressed [58], as well as *POS5* [61]. NADPH availability is estimated by the activity of heterologous NADPH-dependent enzymes. (**B**) Coupling methylation to growth via Mtase selection (adapted from [64]). Black arrows represent the native SAM cycle. SAH stands for S-adenosylhomocysteine, velvet cross represents the inhibition of reaction by gene deletion, green arrows show heterologous reactions. The graphics demonstrate the growth (optical density OD) of yeast strains (the tested strain on the left and the control strain on the right) over time, in the presence (+X) or absence (-X) of the corresponding heterologous Mtase substrate. (**C**) Fe metabolic engineering to enhance heterologous Fe-S enzyme activity (adapted from [65]). The assembly of Fe-S clusters is mediated by the assembly complex. The targeting complex guides Fe-S clusters to the corresponding apoprotein to form Fe-S proteins. Tyw1 binds Fe-S clusters to regulate Fe-S excess in the cell. Truncated Tyw1 (tTyw1) is not able to bind Fe-S clusters. Aft1/2 TFs enhance Fe uptake in the case of Fe deficiency, which is inhibited by the inhibitory complex. Yap5 TF promotes Fe sequestration when in excess. All the represented complexes contain several components omitted here. In the best performing strain, tTyw1 was overexpressed and the inhibitory complex component Bol2 was deleted (Δ*bol2*). The red cross represents the abolishment of inhibitory complex activity.

Figure 5. Strategies of spatial (re)configuration for the metabolic engineering of YCF. (A) Artificial compartmentalization of the triterpene biosynthetic pathway in the peroxisome of *S. cerevisiae*, leading to a significant increase in squalene bio-production (adapted from [69]). **(B)** Artificial compartmentalization of the isoprenoid biosynthetic pathway in the mitochondria of *S. cerevisiae*, leading to a significant increase of geraniol bio-production (adapted from [70]). **(C)** Cell surface engineering of *S. cerevisiae* for the efficient bio-production of ethanol and xylitol from lignocellulosic biomass (adapted from [77, 78, 80]). **(D)** Two different types of artificial metabolons in *S. cerevisiae* (adapted from [32]).

Figure 6. The integrated approach of adaptive laboratory evolution (ALE) to improve YCF, (adapted from [83, 85]**).**
(A) Initial YCFs, obtained by rational ME steps, are cultured in desired growth conditions for an extended period, allowing natural selection to enrich for mutant strains with improved fitness. ALE then occurs via *n* repetitions of propagation of batch cultures, until obtaining the most efficiently evolved YCF (endpoint). **(B)** Enhancement of D-xylose utilization and isobutanol

production by combining the rational DBTL approach and ALE. The ALE experiment was conducted in the presence of 2% D-xylose as a unique carbon source over 12 cycles of cell culture. The pathway has been simplified and the cofactors and certain steps are omitted for easier comprehension. Enzymes written in green color are overexpressed. RE - Reverse Engineering. *Ll - Lactococcus lactis, Ss - Scheffersomyces stipitis, Sc - S. cerevisiae*. Fine-tuning of gene expression - supplementary copies of *LlKivD, ScADH7, ScIvl2, ScIvl3* and *ScIvl5*. **(C)** Evolved strains are characterized for phenotype improvements relative to the parental strain. **(D)** Evolved strains have their DNA sequenced to reveal the adaptive mutations enabling phenotype improvements. **(E)** Augmentation of the typical "Design, Build, Test, Learn" (DBTL) cycle used in ME to generate YCFs. Here, ALE is performed in the continuity of the "Build" step to either rescue a strain that displays decreased fitness due to a perturbation or to optimize a strain after removal or addition of genetic content.

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