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1 **Innovative tools and strategies for optimizing yeast cell factories**

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23

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25 **Keywords**

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

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29

30 **Abstract**

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

41

42

43

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

46

47 Medicinal **natural products (NPs)** (see Glossary) suffer from recurrent shortages, mainly due
48 to the overexploitation of their sources ([https://www.nytimes.com/2019/10/14/health/cancer-](https://www.nytimes.com/2019/10/14/health/cancer-drug-shortage.html)
49 [drug-shortage.html](https://www.nytimes.com/2019/10/14/health/cancer-drug-shortage.html)) [1-3]. While the well-known and long-established uses of synthetic
50 chemistry (SC) allow producing many NPs and their derivatives, at large scales, some
51 compounds such as plant-derived NPs (monoterpene indole alkaloids or MIAs,
52 benzyloquinoline alkaloids or BIAs, etc.) and mammal derived NPs (such as hormones and
53 neurotransmitters) are too complex to be synthesized by SC alone at an industrial scale
54 because of asymmetric carbons, long multi-step biosynthetic pathways, and other factors [3–
55 6]. Although recent progress now allows very complex NPs such as vitamin B₁₂ [7] or
56 strictosidine [8] to be produced synthetically, chemical synthesis is not always the most
57 suitable approach in terms of ecological impact and cost efficiency. Therefore, alternative
58 strategies have been considered to produce these NPs in greater quantities, such as using

59 recombinant microorganisms (Fig. 1, Key Figure). Microorganisms such as yeast have evolved
60 to maintain constant metabolic homeostasis regardless of rapidly changing environmental
61 conditions, so intensively rewiring their metabolism is essential for bioproduction of NPs with
62 high **titer, rate, and yield (TRY)** at scale [5, 6]. Several examples of successful
63 heterogeneous biosynthesis of mammalian and plant NPs exist (such as progesterone [9],
64 hydrocortisone [10, 11], artemisinic acid [12–14], resveratrol [15], strictosidine [2, 16], and (S)-
65 reticuline [17]), but only a few have reached industrial-scale production. For instance, the
66 commercialization of artemisinic acid biosynthesis (a precursor of antimalarial artemisinin),
67 produced at high levels by *Saccharomyces cerevisiae*, did not persist due to poor market
68 demand [14]. In contrast, the major mammal anti-inflammatory hormone hydrocortisone is
69 synthesized by *S. cerevisiae* [10, 11], which is still the main hydrocortisone producer.
70 However, many NPs, particularly highly valuable plant pharmaceuticals (such as anticancer
71 vinblastine and vincristine, or opioids) still cannot be produced by recombinant microorganisms
72 efficiently due to multiple limitations [1-6], which will be discussed in this review. Recently,
73 **metabolic engineering (ME)**, which aims to rewire cellular metabolism, has empowered and
74 accelerated the development of **yeast cell factories (YCFs)** by enhancing the TRY of the bio-
75 produced target compounds, broadening the spectrum of these compounds (e.g., taxol,
76 opioids), increasing the range of substrates (e.g., xylose, arabinose), and enhancing strain
77 physiological properties (e.g., global fitness, stress tolerance) [2–6]. In particular, baker’s yeast
78 (*Saccharomyces cerevisiae*), as well as some non-conventional yeasts with specific features
79 of interest, such as high secretion ability or lipophilic compounds accumulation (including
80 *Pichia pastoris* and *Yarrowia lipolytica* [18, 19]), generally regarded as safe (GRAS) and robust
81 microorganisms, have their genome entirely known and easy to manipulate. In recent
82 decades, remarkable progress has been made in the field of molecular and cell biology of
83 yeast, due to the rapid development of genome sequencing, cell engineering and **synthetic**
84 **biology (SB)** [3–6]. However, many limiting factors remain that make genome manipulation

85 and the construction of YCFs inefficient and time-consuming [5, 6]. Indeed, creating YCFs
86 commonly implies the integration of complex biosynthetic pathways, which requires the high-
87 fidelity assembly of long DNA fragments and sophisticated **genome editing** tools [18, 20, 21].
88 Novel strategies and approaches are continuously developing to facilitate multiplex marker-
89 free genomic integration, DNA assembly and transformation efficiency [4–6, 20, 22].
90 Additionally, integrating heterologous pathways in yeast generally involves fine-tuning gene
91 expression, which has been limited by the availability of characterized native gene regulatory
92 elements, because they are lengthy and unable to cover the required range of the expression
93 strength [23–25]. Multiple approaches aiming at optimizing and extending transcription
94 regulation [26, 27] are still constantly evolving to further broaden the assortment of regulatory
95 elements and overcome their limitations. Moreover, the overexpression of heterologous
96 biosynthetic pathways is frequently responsible for massive stress affecting global cell fitness,
97 which includes the accumulation of toxic intermediates, carbon source competition, and a loss
98 of reducing power (i.e. oxidative stress, and/or unbalanced/competitive use of cofactors) [5, 6,
99 22]. In particular, developing an industrial bioprocess based on YCFs compatible with
100 commercial purposes, with a maximal product TRY and optimal metabolic fluxes of the
101 multiple integrated genes, demands precise control and balance and remains a key goal for
102 already-established recombinant strains [28, 29].

103
104 Furthermore, enzyme and substrate differential subcellular localization [30–32], enzymatic
105 promiscuity, and pathway lateral branches generally decrease substrate availability and
106 metabolic fluxes by hijacking biosynthetic intermediates [33, 34]. As such, formal strategies are
107 also required to redesign metabolic pathways in yeast. This comprehensive review provides an
108 integrative and clear view of the most recent and innovative molecular tools and approaches
109 dedicated to the implementation and improvement of YCFs for the bioproduction of valuable
110 molecules. It will especially emphasize specific challenges associated with already existing

111 methods, and discuss successful examples that highlight the latest tools and advances, and
112 future perspectives.

113

114 **Genome editing tools**

115 In yeast genome editing, homologous recombination and cre/lox-mediated integration have
116 largely been replaced by the CRISPR/Cas9 system, which is still continuously improving to
117 facilitate and accelerate DNA assembly and genome manipulation [18, 21]. Particularly
118 laborious was the assembly of large template DNA (multiple expression cassettes), which is
119 now generally realized *in vivo* via overlapping single-stranded oligonucleotides and
120 homologous recombination (HR), the technique described as DNA assembler [35]. It bypasses
121 multistep and multi plasmid cloning and was successfully employed in an assembly of up to
122 eight-gene biosynthetic pathways in *S. cerevisiae* with the efficiency being inversely correlated
123 to the number of genes [35]. Another limiting factor consisted of multiplex genomic integration,
124 which requires multiple gRNAs. Recently, several novel approaches were developed, aimed to
125 perform multisite targeting and exploiting the simultaneous expression of numerous gRNA
126 using Cas9-expressing yeast strains or all-in-one plasmids (**Fig. 2A**). For instance, in the
127 CasEMBLR method (Cas9-facilitated multi loci integration of assembled DNA parts into *S.*
128 *cerevisiae* chromosomes, in combination with DNA assembler techniques), DNA fragments
129 are amplified by PCR to contain homologous overhangs for further *in vivo* assembly, and
130 marker-free integration was reported to be successful into up to five sites with 50-100%
131 efficiency, which implies both gRNAs-expressing plasmid and Cas9-expressing yeast strain
132 [36, 37]. All-in-one plasmids, such as gRNAs-Cas9 co-expressing vectors with an inducible
133 promoter for Cas9 (also available in constitutive version), enable dissociating cloning from
134 genome editing by yeast pre-transformation with all CRISPR/Cas9 components prior to the
135 introduction of template DNA [38]. Time-effectively, the all-in-one plasmids contain a universal
136 gRNA expression cassette, and the introduction of an appropriate gRNA targeting sequence

137 upon plasmid recircularization takes place *in vivo* in yeast via HR [38]. However, the
138 conventional expression and delivery of multiple gRNAs are delicate, either an individual
139 expression cassette is required for each gRNA, or a common transcript is generated and
140 further cleaved via various strategies into individual gRNAs [39]. Both approaches are limited
141 by the number of gRNAs expressed in the system and the processing efficiency. Recently,
142 alternative strategies were reported. In the GTR-CRISPR (**Fig. 2B**) (tandem gRNA-tRNA array
143 for CRISPR/Cas9) the use of a gRNA-tRNA array within the Cas9-expressing plasmid, and
144 exploiting the endogenous tRNA-processing system, which precisely cleaves the precursor of
145 tRNA and enables the release of single gRNAs from the common gRNA-tRNA transcript,
146 resulted in the simultaneous disruption of 8 genes in *S. cerevisiae* with 87% efficiency [40].
147 Moreover, Lightning GTR-CRISPR, an *E.coli*-free Golden Gate cloning system [41]
148 compatible version can be employed (the Golden Gate reaction is directly transformed into
149 yeast), which was shown to result in 96% and 60% efficiency of correct editing, illustrated
150 through the disruption of four and six genes, respectively [40]. Alternatively, a plasmid-free
151 gRNA delivery method gRNA- Transient Expression System (TES) was described, where
152 gRNA-encoding PCR fragments, composed of the promoter, guiding sequence and gRNA
153 scaffold (two gRNAs are required to cleave both sides of the targeted region), are transformed
154 together with template DNA into Cas9-expressing *S. cerevisiae* strain, which resulted in the
155 substitution of different sized regions of *S. cerevisiae* chromosome 4 by template DNA with 67-
156 100% efficiency [42].

157 In parallel, the CRISPR/Cas9 system has been explored to enhance the efficiency of its
158 components (**Fig. 2B**). In this context Cpf1 (CRISPR from *Prevotella* and *Francisella* 1), a
159 family of class 2/type V CRISPR bacterial endonucleases was found to display several
160 advantages compared to Cas9. For instance, Cpf1 generates sticky ends instead of blunt ends
161 upon DNA cleavage [42], which potentially facilitates template DNA integration. Moreover,
162 Cpf1 possesses both RNAase and DNAase activities and does not need RNAase III, which

163 results in the requirement of only crispr RNA (crRNA) instead of a longer complex of *trans*-
164 activating crispr RNA (tracrRNA) and crRNA [43, 44]. Thus, *S. cerevisiae* strain producing
165 patchoulol was generated using self-cloning Cpf1-crRNA co-expressing plasmid, where
166 singleplex and triplex genomic integrations of *in vivo* assembled template DNA were achieved
167 with 80% and 32% of efficiency, respectively [45].

168 In addition, dCas9-mediated Target-AID (Activation Induced cytidine Deaminase from
169 vertebrate), a synthetic hybrid complex that performs highly efficient C to G and C to T
170 mutations, was successfully used in high throughput loss of function screens [46], which is an
171 appealing application to screen for yeast *de novo* features. On the other hand, the σ
172 sequences, which are a family of repetitive DNA sequences (at least 100 copies) in the *S.*
173 *cerevisiae* genome [47], were used together with the CRISPR/Cas9 system in a novel method
174 of genome shuffling [48]. Cas9, guided by the gRNA targeting the σ sequences, cleaves DNA
175 at multiple sites, thus promoting endogenous DNA repair and mutagenesis [49], which can
176 lead to the improvement of yeast characteristics when cultivated under specific conditions (e.g.
177 thermotolerant *S. cerevisiae* obtained under high-temperature conditions [48]). These
178 approaches demonstrate the application of the CRISPR/Cas9 system beyond gene
179 integration, notably useful in **adaptive laboratory evolution (ALE)**, which will be presented in
180 the last section of this review.

181

182 **Transcriptional (“cis”) and post-transcriptional (“trans”) regulator toolboxes**

183

184 Alteration and regulation of gene expression at the transcriptional level via promoter and
185 terminator elements have been unceasingly considered as a powerful approach. Previously
186 characterized long native elements were initially employed (*S. cerevisiae* pTEF1, pGAL1-10,
187 tCYC1, tADH1, etc) [23–25], which, however, pointed out an extensive necessity of
188 development of novel synthetic units, more compact and functionally diversified. Indeed, the

189 integration of complex multigenic heterologous pathways in yeast mobilizes a promoter and a
190 terminator for each gene, which considerably extends template DNA length, increases the risk
191 of self-recombination, as well as generally demands optimization of gene expression. To
192 address these issues, several regulatory sequence libraries were recently constructed and
193 investigated (**Fig. 3A**). For instance, a library of short 69-bp semi-synthetic promoters,
194 covering an 8.0-fold expression range, was generated from the determined minimal length of
195 *S. cerevisiae* native pTEF1 [51] (**Fig. 3A1**). Moreover, the use of upstream activating
196 sequences (UAS) expanded the library to reach a 20-fold expression range and a maximal
197 length of 130 bp. Importantly, some of the pTEF1 variants demonstrated expression strength
198 comparable to native pTEF1 and pPGK1. Another library of *Y. lipolytica* semi-synthetic
199 promoters was generated, also based on the promoter core region between the TATA-box and
200 the transcriptional starting site (TSS) upstream the 5' untranslated region (5'UTR) sequence
201 [51] (**Fig. 3A2**). Artificial sequences of 30 bp in length were designed (various combinations of
202 T-rich and G/C-rich fragments) and substituted in *Y. lipolytica* strong native pEXP1 and pGDP
203 to assess expression levels of crtY enzyme and, consequently, conversion of lycopene to β -
204 carotene. The results demonstrated a population of novel *Y. lipolytica* promoters showing an
205 up to a 5.5-fold increase in lycopene conversion. On the other hand, the impact of 10-bp
206 terminator linker 1 (the sequence between the efficiency element and the positioning element)
207 on gene expression was investigated via synthetic terminator library in *S. cerevisiae* [52] (**Fig.**
208 **3A3**). A 6.0-fold expression range was achieved and it was demonstrated that sequences with
209 low GC content and enriched in T were conferring higher levels of expression.

210 In addition to constitutive regulation of gene expression, **metabolic biosensors** play a central
211 role in metabolic rewiring and the optimization of productivity in YCFs. For instance,
212 **Metabolite responsive allosteric transcription factors (aTFs or MRTFs)** from bacteria
213 interact with their corresponding metabolites prior to acting as repressors or activators of
214 transcription [53]. To alter transcription, MRTFs bind promoter-specific sequences and,

215 therefore, restrict or facilitate the access to RNA polymerase [53]. Thus, MRTFs enable
216 dynamic control of heterologous gene expression and, consequently, regulate metabolic
217 activity. However, the engineering of MRTFs in eukaryotic cells remains more than
218 challenging. Recently, a novel strategy has been reported and aimed to generate user-defined
219 biosensors via evolution-guided toggled selection (directed biosensor evolution and library
220 construction, followed by the selection of mutants via alternating conditions according to the
221 set criteria), based on the mutagenesis of aTF effector binding domain (EBD) and
222 Fluorescence-Activated Cell Sorting (FACS)-mediated selection [54] (**Fig. 3B1**). Thus, a
223 variety of *de novo* features were identified, such as inversion of function and change of
224 specificity, as well as modification of dynamic and operational range. Another approach
225 focused on the optimization of biosensor reporter promoters in a binding site-dependent
226 manner [55] (**Fig. 3B2**). The constructed synthetic promoter libraries (from native *pTEF1* and
227 truncated *pCYC1*) covered all the possible positions for introducing aTFs binding site within
228 promoter core region, which resulted in the identification of function-related positions for most
229 of the screened repressor or activator type aTFs [55]. The complementary aspect of gene
230 expression regulation via promoters is related to the carbon source of yeast metabolism. Given
231 that multiple commonly used native promoters are associated with glycolytic genes and are
232 less effective under glucose starvation [25], novel promoters, activated upon diauxic shift, are
233 required to maintain constitutive gene expression (or to disassociate growth from production if
234 required). Recently, **carbon source response elements (CSRE)** of gluconeogenic promoters
235 were introduced upstream UAS of *S. cerevisiae pTDH3*, substituting the binding site of
236 glycolytic genes transcriptional activator Gcr1 and surrounding neutral sequence [56] (**Fig.**
237 **3C**). This approach demonstrated strong induction of reporter Yellow Fluorescent Protein
238 (YFP) in the low-glucose medium instead of a glucose-rich medium, which was validated in a
239 vanillin- β -glucoside-producing yeast strain. Indeed, when glycolytic promoters were employed,
240 the production of vanillin- β -glucoside took place mostly during the ethanol phase leading to the

241 accumulation of the cytotoxic intermediate protocatechuic acid (PCA). The use of generated
242 gluconeogenic promoters to control the expression of enzymes converting PCA to vanillin- β -
243 glucoside showed an enhanced yeast growth, lower accumulation of PCA, and conserved
244 vanillin- β -glucoside bioconversion [56]. Taken together, these advances are broadening the
245 collection of available gene regulation elements and deliver compelling guidance to
246 combinatorial approaches for metabolic rewiring in YCF.

247

248 **Rewiring of primary metabolism**

249

250 The strategies to enhance TRY imply the optimization of host primary metabolism, which
251 includes the modification of native metabolic fluxes and coupling/decoupling growth with
252 production. Sufficient availability of the NADPH cofactor, required by numerous enzymes, in
253 some cases (excluding, for instance, hydrocortisone biosynthesis [10]) stands as a limiting
254 factor (e.g. *S. cerevisiae* 3-hydroxy-3-methylglutaryl-CoA reductase Hmg1, *S. cerevisiae*
255 squalene synthase Erg9 etc) [57, 58], given that glucose is dispatched in both glycolysis and
256 pentose phosphate pathway (PPP) upon consumption [59]. Several successful approaches
257 have been implemented to enhance NADPH (re)generation (**Fig. 4A**) and, therefore,
258 heterologous/homologous production. For instance, a number of *S. cerevisiae* genes involved
259 in NADPH synthesis were deleted or overexpressed to assess the effect on NADPH
260 concentration in the cytosol and heterologous protopanaxadiol (PPD) production from
261 endogenous squalene [57]. In the best performing PPD-producing *S. cerevisiae* strain, the
262 deletion of NADH-generating *ALD2* and overexpression of NADPH-generating *ALD6* aldehyde
263 dehydrogenases-encoding genes, involved in ethanol metabolism, resulted in a 1.3-fold
264 increase of NADPH cytosolic concentration and a 4.5-fold increase in PPD production [57].
265 Likewise, the positive effect of the overexpression of the *Ald6*-encoding gene was
266 demonstrated in recombinant noscapine-producing *S. cerevisiae* [61]. The overexpression of

267 *S. cerevisiae* full-length mitochondrial or truncated cytoplasmic NADH kinase Pos5, which
268 catalyzes NADPH-generating reactions, led to the 9.0-fold and 7.0-fold enhancement of
269 homologous squalene production respectively [58]. Similarly, the overexpression of truncated
270 cytoplasmic Pos5 in antibody fragment-expressing *P. pastoris* was shown to significantly
271 increase the production of recombinant protein [61].

272 Another important cofactor in cellular metabolism is *S*-adenosylmethionine (SAM), which is a
273 donor of the methyl group upon methylation catalyzed by SAM-dependent methyltransferases
274 (Mtases) [62]. Methylation, being required by a wide range of cellular processes, is used in
275 biotechnological approaches for the heterologous production of valuable methylated
276 compounds [63]. Engineering of SAM-dependent Mtases has been challenging, mostly due to
277 the lack of efficiency and consistency. Recently, methylation was coupled to growth by
278 combining enhanced Mtase activity and cysteine biosynthesis [64] (**Fig. 4B**), which was
279 achieved via Mtase adaptive laboratory evolution *in vivo* and deletion of several *S. cerevisiae*
280 genes. On the other hand, certain enzymes require specific chemical elements for their
281 formation and activity such as the bacterial xylonate dehydratase (XylID, involved in xylose
282 metabolism) that contains iron-sulfur (Fe-S) cluster [65]. Although *S. cerevisiae* Fe metabolism
283 was not reported to be limiting for bovine Fe-S adrenodoxin and hydrocortisone biosynthesis
284 [10], the folding and the activity of several bacterial Fe-S enzymes were reported to be
285 insufficient in yeast [66–68], still being a key concern in the functional expression of bacterial
286 Fe-S proteins. Recently, Fe metabolism of yeast cytosolic Fe-S cluster machinery was
287 modified to enhance Fe uptake, XylID generation, and 1,2,4-butanetriol production [65] (**Fig.**
288 **4C**). In the best performing recombinant *S. cerevisiae* strain, the overexpression of truncated
289 Tyw1 protein (tTyw1), unable to bind and sequester Fe-S clusters, and the deletion of one of
290 the components of negative regulation of Fe uptake, resulted in 6.0-fold and 1.4-fold higher
291 XylID activity and 1,2,4-butanetriol production, respectively [65].

292

293 **Spatial reconfiguration strategies**

294 The development of valuable compound-producing YCFs usually involves complex enzymatic
295 pathways, requiring specific physicochemical conditions, substrates and cofactors, potentially
296 affected by adverse side reactions or toxic intermediates [6, 20]. To meet these challenges,
297 several approaches based on the spatial rearrangement of the desired pathway(s) have
298 emerged, from the metabolic engineering of organelles (MEO) to the construction of artificial
299 **metabolons** [30–32]. Recently, successful artificial compartmentalization of the triterpene
300 biosynthetic pathway in the peroxisome of *S. cerevisiae* allowed producing a high
301 concentration of squalene (**Fig. 5A**) revealing this organelle as a promising site for the
302 biosynthesis and storage of terpene compounds [69]. In particular, the rapid and highly
303 efficient protein import machinery of peroxisomes, along with their high plasticity (number and
304 size dynamically adjusted according to the physiological state of the cells), combined to a
305 channeling effect insured by the close vicinity of overexpressed heterologous biosynthetic
306 enzymes, make this subcellular compartment an ideal target for improvement of YCFs.

307 Previously, the successful bioproduction of hydrocortisone in yeast elegantly showed the
308 flexibility of yeast in accommodating the relocalization of membrane-bound enzymes to a
309 different subcellular compartment, without affecting the final titer of hydrocortisone produced
310 [10, 11]. More recently, the successful artificial compartmentalization of isoprenoid
311 biosynthesis into the mitochondria was achieved (**Fig. 5B**), with an enhanced supply of acetyl-
312 CoA and tricarboxylic acid cycle intermediates [70]. However, as mitochondrion is gathering
313 crowds of essential proteins involved in respiration, the compartmentalization of heterologous
314 biosynthetic pathways in this organelle might be, in some cases, responsible for metabolic
315 stresses. In MEO, important physiological parameters such as the size and the biogenesis of
316 organelles can increase the physical space available for enzyme encapsulation and storage of
317 metabolites [71–74]. For example, increasing the size of lipid droplets, by modulating
318 triacylglycerol metabolism, allowed a 1.25-fold increase in lycopene bio-production in a

319 recombinant strain of *S. cerevisiae*, correlated to the improved storage capacity of hydrophilic
320 lycopene [74]. Similarly, the overexpression of a key ER size regulatory factor gene, *INO2*,
321 allowed a significant increase of the surface of the ER [73], associated with a drastic
322 augmentation of production of P450s [71-73], and lead to an 8-fold increase in PPD bio-
323 production, along with a 7.1-fold increase of protein secretion, emphasizing the crucial role of
324 ER in protein synthesis and folding, to circumvent potential metabolic constraints [73, 75].

325 In parallel to MEO, another approach has emerged allowing the display of enzymes directly at
326 the surface of the yeast cells (**Fig. 5C**) [76]. The **cell surface engineering (CSE)** approach
327 enables the generation of the whole cell catalysts to achieve the hydrolysis of numerous
328 substrates, including lignocellulosic biomass, consecutively converted by fermentation into a
329 variety of valuable compounds such as ethanol and xylitol [31, 76–78]. In brief, CSE consists
330 of targeting heterologous enzymes to the cell wall, through the secretion pathway, to expose
331 their catalytic sites toward the extracellular environment. The enzymes can be displayed either
332 individually or gathered in association with a protein-based scaffold to form the **cellulosome**, a
333 multi-enzymatic complex structure capable of accommodating up to 63 enzymes for the largest
334 one reported to date [31, 78]. One of the main challenges in CSE resides in controlling the
335 efficiency of the display, as well as the anchorage position of the target protein within the cell
336 wall. In the case of the bio-production of xylitol from lignocellulose by CSE, the uptake of
337 xylose across the membrane of the cells constitutes a critical point, as the bioconversion
338 reaction requires NADH and cannot be achieved at the surface of the cells [77, 79].
339 Interestingly, the co-expression of a homologous maltose transporter (*ScMal11*) along with a
340 beta-glucosidase (*AaBgl*) degrading cello-oligosaccharides (e.g. cellobiose) on the cell surface
341 of *S. cerevisiae* allowed to enhancing xylitol production from the co-utilization of
342 cellobiose/xylose contained in lignocellulose [80].

343 To prevent the accumulation of toxic/unstable intermediates as well as the loss of the desired
344 intermediates, another interesting option has emerged based on the spatial (re)arrangement of

345 enzymes into artificial metabolon, ensuring substrate channeling for a continuous metabolic
346 flux within the YCF [32, 81]. Two main approaches enable constructing such artificial
347 metabolons: one consisting of a direct fusion of the enzymes to each other (protein linker), and
348 one relying on the interaction of enzymes with protein (or nucleic acid)-based scaffolds (**Fig.**
349 **5D**). While protein fusion is considered as the easiest way to enhance substrate channeling,
350 this approach may alter the structure of the enzymes and is always restricted to a very limited
351 (two to three) number of enzymes. Nevertheless, the over-expression of an engineered
352 tridomain enzyme (CrtB, CrtI, CrtY) harboring the full β -carotene biosynthetic pathway lead to
353 an improved bio-production of the pigment in *S. cerevisiae* highlighting the potential of this
354 approach for the improvement of YCFs [81]. On another hand, artificial metabolon using
355 protein-based scaffold technologies allows the gathering of several enzymes in close vicinity
356 through affinity binding, leading to significantly improved xylose utilization and resveratrol bio-
357 production in *S. cerevisiae* and to decrease the accumulation of by-product xylitol [82].

358

359 **Adaptive laboratory evolution strategies**

360

361 Besides pathway engineering and metabolic flux rewiring approaches, the development of
362 robust YCFs is crucial for industrial application purposes, due to harsh culture conditions and
363 frequent limiting toxicity of intermediates and products [83, 84]. Adaptive laboratory evolution
364 (ALE) relies on yeast adaptation capacity allowed by multiple DNA recombination events and
365 high genomic plasticity, utterly difficult to achieve through the rational engineering approach
366 alone (**Fig. 6A**) [83]. For instance, ALE led to improved xylose utilization ability in *S. cerevisiae*
367 along with an enhanced isobutanol bioproduction due to point mutations in the *CCR4* and *TIF1*
368 genes, and fine-tuning of gene expression in the evolved strain (**Fig. 6B**) [85]. Associated with
369 high throughput screening methodologies [84, 86], ALE constitutes a powerful approach
370 allowing speeding up the conception of highly efficient recombinant YCFs (**Fig. 6C-E**). In

371 particular, ALE allows overcoming numerous physiological limitations (i.e. thermotolerance,
372 osmotic stress, low pH, toxicity, etc) [48, 84–92] as well as to complement the rational
373 engineering approaches presented in the above sections, and providing new targets for the
374 next round of rational design [64, 83–86, 92–102]. Noteworthy, the use of CRISPR/Cas9
375 system in combination with ALE approach enabled the generation of evolved YCF with
376 improved thermotolerance, as mentioned in the “*Genome editing*” section of this review [48].
377 Other striking studies, such as growth-coupling strategies associated to ALE, can be cited
378 [64, 103]. In particular, when the production of the essential amino acid cysteine was tied to
379 the activity of methyltransferases, ALE was used to select for both *E. coli* and *S. cerevisiae*
380 strains with mutations leading to 2-fold increases in heterologous methyltransferase activity
381 [64]. In this study, adaptive mutations were forced to preferentially target the methyltransferase
382 activity bottleneck which was limiting for growth-rate. This approach allowed to significantly
383 improve activity of both N- and O-type methyltransferases, as illustrated in the “*Rewiring of*
384 *primary metabolism*” section of this review.

385 In addition, ALE virtually allows to improve the “**Design, Build, Test, Learn**” (DBTL) cycle
386 classically used in ME to generate YCFs (**Fig. 6E**), when performed in the continuity of the
387 “Build” step for rescuing/optimizing a strain with decreased fitness [83]. Nevertheless, given
388 that that evolution of the strains often drives the population towards a low-production high-
389 fitness phenotype, which constitutes an important limitation of ALE, an extensive effort is still
390 required to overcome this problem [83–85, 103, 104]. In that sense, combining ALE with multi-
391 omic technologies is emerging as the most promising and efficient approach in YCFs
392 engineering [48, 64, 84, 86, 95, 102, 104, 105].

393

394

395 **Concluding remarks: Future Challenges and Directions**

396

397

398 Using synthetic biology to implement YCFs is a promising approach to produce very complex
399 compounds (including vinblastine and vincristine, among many others) that are almost
400 impossible to produce by synthetic chemistry alone because of challenges like asymmetric
401 carbons. Although several molecular tools (e.g., halogenases) have been characterized in
402 marine bacteria and plants, reports on bioproduction of halogenated NPs in yeast is scarce
403 [106-108]. This kind of subtle modification (such as chlorination or fluorination) remains a
404 future challenge for engineering YCFs to generate new-to-nature compounds with high
405 pharmaceutical interest (see Outstanding Questions) [1, 2, 106-109]. Recently, an indirect way
406 to obtain such compounds was reported, consisting of incorporating precursor derivatives (in
407 this case, halogenated tyrosine) to produce S-reticuline derivatives [60]. Another recent study
408 elegantly showed the bioproduction of halogenated oxo-(2-aminophenyl) and quinoline
409 scaffolds in *S. cerevisiae*, by overexpressing regiospecific L-tryptophan halogenases [109].
410 Therefore, SC is still indispensable to generate valuable fluorinated or chlorinated NPs
411 derivatives, usually presenting much higher pharmacodynamic characteristics, and a larger
412 demand in drug industries. One example is dexamethasone, the fluorinated derivative of
413 hydrocortisone, which is 40 times more potent than hydrocortisone and far more stable *in vivo*.
414 In that sense, a strong complementarity between SB and SC exists, while SB is a highly
415 sustainable and ecological way to produce platform compounds, SC can be employed in
416 downstream fine-tuning and upstream precursor-directed yeast-mediated biosynthesis
417 (bioconversion, [110]). Despite some technical limitations, yeast cells represent a useful
418 eukaryotic tool suitable for the bio-production of valuable and complex molecules. Meanwhile,
419 constant progress in SB has accelerated the industrial applications of YCFs [1–6]. Besides
420 model yeasts such as *S. cerevisiae*, several non-conventional chassis present increasing
421 interest in ME applications due to their unique features [18, 19]. While SC has made huge
422 progress in recent years, including the recent syntheses of molecules as complex as Vitamin
423 B₁₂ [7] or strictosidine [8], tailoring ideal YCFs that can produce large-scale NPs as well as

424 their derivatives is essential to establish next-generation bio-foundries as cost- and time-
425 efficient alternatives. Such progress in the field of SB will constitute an invaluable chance to
426 further achieving a better and more sustainable future, in agreement with the Sustainable
427 Development Goals (SDGs) of the United Nations
428 (<https://www.un.org/sustainabledevelopment/sustainable-development-goals/>). The coming
429 decade is undoubtedly going to be full of exciting improvements in the YCF technology,
430 leading to the ultimate convergence of SC and SB: the production of a truly infinite palette of
431 complex molecules.

432

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447 space limitations.

448

449

450 Figure Legends

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

460

461 Figure360. An author presentation of Figure 1.

462

463 **Figure 2. Advanced genome editing tools in yeast (A)** DNA assembly and CRISPR/Cas9-
464 mediated yeast transformations. Cas9 is expressed in yeast (plasmid-encoded under
465 constitutive or inducible promoter, according to user needs). This is achieved either in tandem
466 with gRNA(s) [38], followed by the Cas9-gRNA(s)-expressing strain transformation with
467 template DNA, or yeast pre-transformation with Cas9-expressing plasmid only, further followed
468 by the transformation of Cas9-expressing strain with template DNA and gRNA(s) plasmid [36,
469 37]. A recent gRNA delivery method transient expression system (TES) was described, where
470 the PCR fragments encoding the gRNAs (and containing the promoter, the guiding sequence
471 and the gRNA scaffold) are transformed together with template DNA into Cas9-expressing
472 yeast strain [42]. The template DNA (multiple expression cassettes, composed of promoter
473 and terminator for each gene of interest) is assembled *in vivo* via overlapping oligonucleotides
474 (here represented as colored lines at the extremities of expression cassettes, black lines
475 represent the so-called Arms - the sequences homologous to the specific yeast genomic
476 regions) and homologous recombination (HR) [36, 37]. The schematic example of a single
477 expression cassette is shown. **(B)** Advances in CRISPR/Cas9 system. The GTR-CRISPR
478 (tandem gRNA-tRNA array for CRISPR/Cas9) is based on the use of a gRNA-tRNA array
479 encoded in a plasmid with Cas9, and the native tRNA-processing system (the precursor of
480 tRNA is cleaved, which releases the individual gRNAs from the common transcript) [40]. Cpf1
481 (CRISPR from *Prevotella* and *Francisella* 1 endonuclease) demonstrates several advantages
482 compared to Cas9 (generation of sticky ends, RNase and DNase activity, requirement of only
483 crRNA) [44]. dCas9-mediated Target-AID (Activation Induced cytidine Deaminase from
484 vertebrate) is a synthetic hybrid complex, which performs C to G and C to T mutations with
485 high accuracy [46].

486

487 **Figure 3. New tools for the regulation of gene expression in yeast. (A)** Generation of
488 synthetic gene regulatory element libraries. Promoter (P) core region between the TATA box
489 and the transcriptional starting site (TSS) was mutated. 1) The determined minimal sequence
490 of *S. cerevisiae* pTEF1 core region was mutated. The variants, with or without UASs, were
491 screened by measuring yECitrine (yECit, yellow-green fluorescent protein) fluorescence [50].
492 2) The artificial sequences enriched in T and/or G/C were substituted in *Y. lipolytica* pEXP1
493 and pGDP core regions. The screening - colony-color spectrum due to crtY activity
494 (accumulation of carotene pigments) [51]. 3) In the *S. cerevisiae* terminator library terminator
495 (T) linker 1 sequence between the efficiency element (EE) and the positioning element (PE)
496 was randomized. The screens were done using green fluorescent protein (GFP) fluorescence
497 as a reporter [52]. **(B)** Engineering of metabolite sensing and downstream regulation in *S.*
498 *cerevisiae*. 1) The method enables the design of user-defined MRTFs by random mutagenesis
499 of the MRTF effector binding domain (EBD) upstream DNA binding domain (DBD). The

500 selection of MRTFs with *de novo* features is GFP fluorescence-activated cell sorting (FACS) -
501 based. ON state - dark green (presence of inducer), OFF state - light green (control medium).
502 The dashed line rectangle - variant selection, the purple background - control strain not
503 expressing MRTF. The criteria for selection vary according to user needs [54]. 2) Activator- or
504 repressor-type MRTF binding site (BS) were inserted in front of every nucleotide of native
505 pTEF1 or truncated pCYC1 core regions to generate MRTF operator library [55]. (C)
506 Construction of synthetic gluconeogenic promoters in yeast [56]. Native promoters were
507 reshaped from glycolytic to gluconeogenic via substitution of the glycolytic genes
508 transcriptional activator Gcr1 BS by carbon source response elements (CSRE) of native
509 gluconeogenic promoters. sP - synthetic promoter, purple and black circles/curves - ethanol
510 and glucose, respectively. Overlapping of curves - diauxic shift.
511

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

532 **Figure 5. Strategies of spatial (re)configuration for the metabolic engineering of YCF.**
533 (A) Artificial compartmentalization of the triterpene biosynthetic pathway in the peroxisome of
534 *S. cerevisiae*, leading to a significant increase in squalene bio-production (adapted from [69]).
535 (B) Artificial compartmentalization of the isoprenoid biosynthetic pathway in the mitochondria
536 of *S. cerevisiae*, leading to a significant increase of geraniol bio-production (adapted from
537 [70]). (C) Cell surface engineering of *S. cerevisiae* for the efficient bio-production of ethanol
538 and xylitol from lignocellulosic biomass (adapted from [77, 78, 80]). (D) Two different types of
539 artificial metabolons in *S. cerevisiae* (adapted from [32]).
540

541 **Figure 6. The integrated approach of adaptive laboratory evolution (ALE) to improve**
542 **YCF, (adapted from [83, 85]).**

543 (A) Initial YCFs, obtained by rational ME steps, are cultured in desired growth conditions for an
544 extended period, allowing natural selection to enrich for mutant strains with improved fitness.
545 ALE then occurs via *n* repetitions of propagation of batch cultures, until obtaining the most
546 efficiently evolved YCF (endpoint). (B) Enhancement of D-xylose utilization and isobutanol
547 production by combining the rational DBTL approach and ALE. The ALE experiment was

548 conducted in the presence of 2% D-xylose as a unique carbon source over 12 cycles of cell
549 culture. The pathway has been simplified and the cofactors and certain steps are omitted for
550 easier comprehension. Enzymes written in green color are overexpressed. RE - Reverse
551 Engineering. *Ll* - *Lactococcus lactis*, *Ss* - *Scheffersomyces stipitis*, *Sc* - *S. cerevisiae*. Fine-
552 tuning of gene expression - supplementary copies of *LlKivD*, *ScADH7*, *ScIv12*, *ScIv13* and
553 *ScIv15*. **(C)** Evolved strains are characterized for phenotype improvements relative to the
554 parental strain. **(D)** Evolved strains have their DNA sequenced to reveal the adaptive
555 mutations enabling phenotype improvements. **(E)** Augmentation of the typical “Design, Build,
556 Test, Learn” (DBTL) cycle used in ME to generate YCFs. Here, ALE is performed in the
557 continuity of the “Build” step to either rescue a strain that displays decreased fitness due to a
558 perturbation or to optimize a strain after removal or addition of genetic content.

559

560 **Glossary**

561

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

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

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

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

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

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

579 **Global fitness:** physiological state of living cells at a given time.

580 **Metabolic biosensors:** biomolecules able to detect/respond to specific metabolites within
581 metabolic pathways.

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

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

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

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

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

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

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

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

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

602

603

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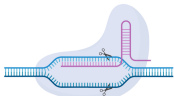
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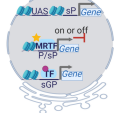
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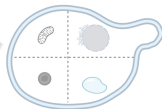
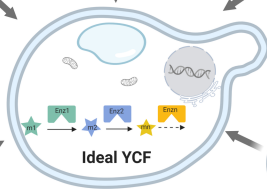
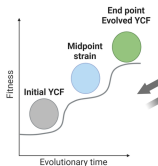
Genome editing tools



'Cis-trans' regulation

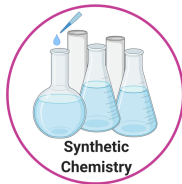


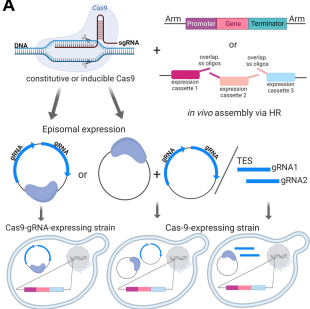
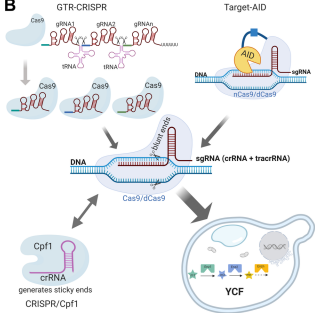
Rewiring of primary metabolism

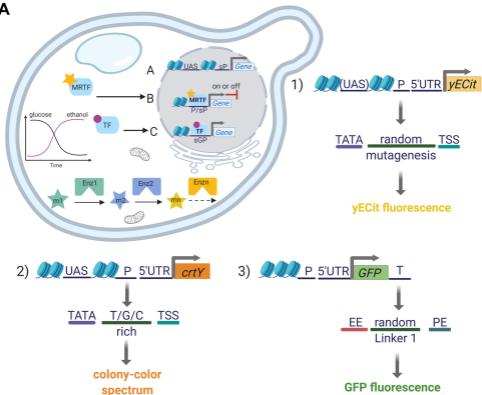
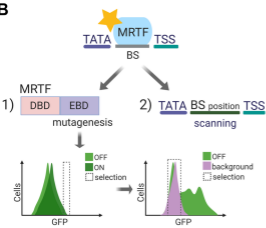
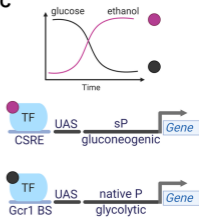


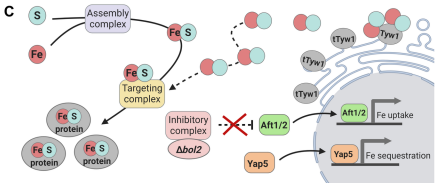
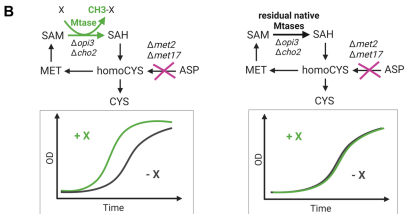
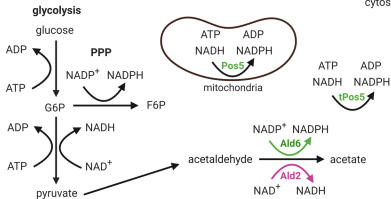
Adaptive Laboratory Evolution

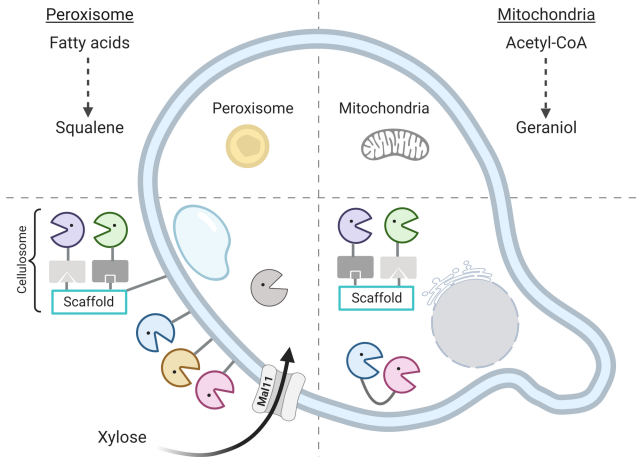
Spatial reconfiguration



A**B**

A**B****C**



A Artificial Peroxisomal Compartmentation **B Artificial Mitochondria Compartmentation****C Cell Surface Engineering****Artificial Metabolons D**

