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Under pressure: evolutionary engineering of yeast strains for improved performance in fuels and chemicals production

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Evolutionary engineering, which uses laboratory evolution to select for industrially relevant traits, is a popular strategy in the development of high-performing yeast strains for industrial production of fuels and chemicals. By integrating whole-genome sequencing, bioinformatics, classical genetics and genome-editing techniques, evolutionary engineering has also become a powerful approach for identification and reverse engineering of molecular mechanisms that underlie industrially relevant traits. New techniques enable acceleration of *in vivo* mutation rates, both across yeast genomes and at specific loci. Recent studies indicate that phenotypic trade-offs, which are often observed after evolution under constant conditions, can be mitigated by using dynamic cultivation regimes. Advances in research on synthetic regulatory circuits offer exciting possibilities to extend the applicability of evolutionary engineering to products of yeasts whose synthesis requires a net input of cellular energy.

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Introduction

Genetically engineered yeast strains are increasingly used to produce commodity chemicals and biofuels such as succinic acid, isoprenoid-based hydrocarbons, isobutanol and ethanol [1–3]. Strain improvement is essential to achieve the product yields and productivities required for economic viability of these processes. Especially in *Saccharomyces cerevisiae*, CRISPR/Cas9-mediated genome editing and DNA-assembly methods enable fast and precise introduction of targeted genetic modifications [4,5]. Evolutionary engineering, also known as adaptive laboratory evolution, is a complementary strain-improvement strategy, which exploits plasticity of microbial genomes by designing and applying cultivation regimes that confer a specific selective advantage to mutants with an industrially relevant trait [6,7]. The selective advantage of such mutants relative to other cells in the population can be based on a higher specific growth rate, lower death rate and/or increased retention in the culture (e.g. by sedimentation or biofilm formation, Table 1; [8,9]). Evolutionary engineering has been intensively applied to wild-type and engineered yeast strains, for example to improve stress tolerance, substrate-consumption kinetics and catabolic product-formation rates (Table 1). This paper discusses recent developments and key challenges in evolutionary engineering for improved performance of yeasts in the industrial production of fuels and chemicals.

Evolutionary engineering: batch and continuous cultivation strategies

Serial transfer in simple shake flasks or tubes (Figure 1) remains a powerful approach for yeast evolutionary engineering. Especially when each new cycle is inoculated from an exponentially growing culture, while maintaining a constant or continually increasing selective pressure, serial transfer selects for mutants with a higher maximum specific growth rate (μ_{max}). Serial-transfer experiments have yielded yeast strains with improved stress tolerance (e.g. to high product and inhibitor concentrations, high temperature, low pH) and with improved rates of substrate consumption and/or catabolic product formation (Table 1). In fundamental research, automated serial transfer enabled massive parallel yeast evolution experiments [10]. Recent studies on evolutionary engineering of bacteria [11–13] underline the potential of automation for intensifying yeast evolutionary engineering.

Sequential batch reactors (SBRs) combine automation of repeated batch cultivation with accurate control of process parameters. Automated empty–refill cycles can, for example, be based on the actual CO_2 output, where a decrease of the CO_2 concentration in the off gas indicates nutrient depletion and triggers the onset of the next cycle [8]. After emptying, a small remaining fraction of the culture then acts as inoculum for the next cycle (Figure 1). Attention is therefore required to prevent accumulation of fast-sedimenting mutants that bypass selection for fast growth [8].

Evolutionary engineering can also be performed in well-established systems for selection of fast-growing mutants

Table 1

Recent applications of various strategies for evolutionary engineering in *Saccharomyces cerevisiae*. *Abbreviations*: WGS, whole-genome sequencing; SNP, single-nucleotide polymorphism; HMF, hydroxymethylfurfural.

Target	Strategy	Evolution time	Evolved phenotype	Genotype analysis	Proven causal mutations	Reference
Serial transfer in shake flasks						
Faster glycerol utilization	Serial transfer in synthetic medium with glycerol as sole carbon source	55 generations	Growth on glycerol (0.12–0.13 h^{-1})	3 Rounds of backcrossing and WGS of independently evolved mutants	SNPs in *GUT1* and *UBR2*	[38]
3-Hydroxypropionic acid (3-HP) tolerance	Serial transfer in complex medium supplemented with 3-HP at pH 3.5	Circa 200 generations	Growth in YPD medium at pH 3.5 with 50 g L^{-1} 3-HP (0.18–0.20 h^{-1})	WGS of independently evolved mutants	Mutations in *SFA1*	[35•]
High-temperature tolerance	Serial transfer in synthetic medium at 39.5 °C	326–375 generations	1.5–2-fold increased growth rate at 40 °C	WGS of independently evolved mutants	Early stop codon in *ERG3*	[43]
Evolution of HXT transporter into glucose-insensitive xylose transporter	Serial transfer of a strain unable to metabolize glucose on synthetic medium with xylose as carbon source in the presence of increasing concentrations of glucose	7 transfers	Growth on 10 g L^{-1} xylose in the presence of up to 50 g L^{-1} glucose	Sequencing of *GAL2, HXT5*, *HXT7* genes (independent evolution experiments).	SNPs in *GAL2*, *HXT5* and *HXT7*	[24•]
Sequential batch reactor (SBR) cultivation						
Full biotin prototrophy	SBR cultivation on synthetic medium without biotin. Empty-refill cycles based on CO_2 concentration in off gas	11 transfers	32-fold increased growth rate in the absence of biotin (0.32 h^{-1})	WGS	19-fold amplification of *BIO1* gene and inactivation of *TPO1*	[16]
Fast biomass sedimentation	Anaerobic SBR cultivation with glucose or galactose as carbon source. Effluent pipe for empty-refill cycles above bottom of bioreactor. Empty-refill cycles based on CO_2 concentration in off gas	Circa 500 and circa 900 generations	Complete sedimentation after 5 min of static incubation	WGS of independently evolved mutants	Whole-genome duplication and mutations in *ACE2*	[8]
Continuous cultivation						
Full biotin prototrophy	Accelerostat with feedback-controlled dilution rate based on CO_2 concentration in the off gas on synthetic medium without biotin	48–77 days	25–36-fold increased growth rate in the absence of biotin (0.25–0.36 h^{-1})	WGS of independently evolved mutants	8–42-fold amplification of *BIO* genes inactivation of *PDR12* and *TPO1*	[16]
Improved xylose fermentation in the presence of lignocellulosic inhibitors	Mutagenesis and anaerobic chemostat cultivation on non-detoxified straw hydrolysate with 20 g L^{-1} xylose	100 generations	7.5-fold reduction of lag phase and complete removal of HMF, furfural and acetic acid in 24 h (0.12 h^{-1})	Not performed	Not performed	[52]

Table 1 (*Continued*)

Target	Strategy	Evolution time	Evolved phenotype	Genotype analysis	Proven causal mutations	Reference
Improved ethanol tolerance	Aerobic turbidostat cultivation on complex medium with gradually increasing concentrations of ethanol (6% up to 12%)	200 generations	Up to 2.5-fold increased fitness in medium containing 9% ethanol and growth in the presence of 12% ethanol	WGS of independently evolved mutants and cultures	Increased ploidy and mutations in *PRT1*, *MAX67* and *VPS70*	[40]
Dynamic selection regimes						
Constitutive tolerance to high concentrations of acetic acid	Serial transfer in synthetic medium with and without acetic acid (alternating) in shake flasks	50–55 transfers	Growth in synthetic medium containing >12.5 g L^{-1} acetic acid at pH 4.5	WGS of independently evolved mutants and backcrossing	Mutations in *ASG1*, *ADH3*, *SKS1* and *GIS4*	[37•]
Synthetic selection circuits						
Strains with improved aromatic amino acid (AAA) pathway flux.	Mutagenesis and serial transfer of a strain with an AAA-biosensor (pARO9-KanNeo) in the presence of increasing levels of G418 and 4-fluorophenylalanine (4-FP)	Circa 11 transfers	Growth in the presence of 1 g L^{-1} G418 and 7.5 mM 4-FP and a 4–9-fold increased AAA pool.	Not performed	Not performed	[77••]

such as pH-auxostats, turbidostats [14,15] and related continuous-cultivation set-ups (Figure 1). Glucose-grown continuous cultures, whose dilution rates were feed-back controlled based on on-line CO_2-production measurements, were recently used for selecting fast-growing biotin-prototrophic *S. cerevisiae* strains (Table 1) [16]. In another example, *S. cerevisiae* chemostat cultures grown on a synthetic glucose–acetic acid–ammonium medium without pH control, in which ammonium consumption led to acidification, were used to automatically maximize selective pressure for tolerance to acetic acid, a key inhibitor of yeast performance in lignocellulosic hydrolysates. After 400 generations of selective growth, the permissive acetic-acid concentration of evolving cultures had increased by three fold [17]. In industrial batch processes, low substrate affinity (μ_{max}/K_s; [18]) causes extended fermentation times. Nutrient-limited chemostat cultivation (Figure 1), which strongly selects for mutants with an improved affinity for the growth-limiting nutrient, has been extensively applied to improve affinity of wild-type [19,20] and engineered yeasts for various carbon sources, including D-xylose and sucrose (Table 1) [21–23].

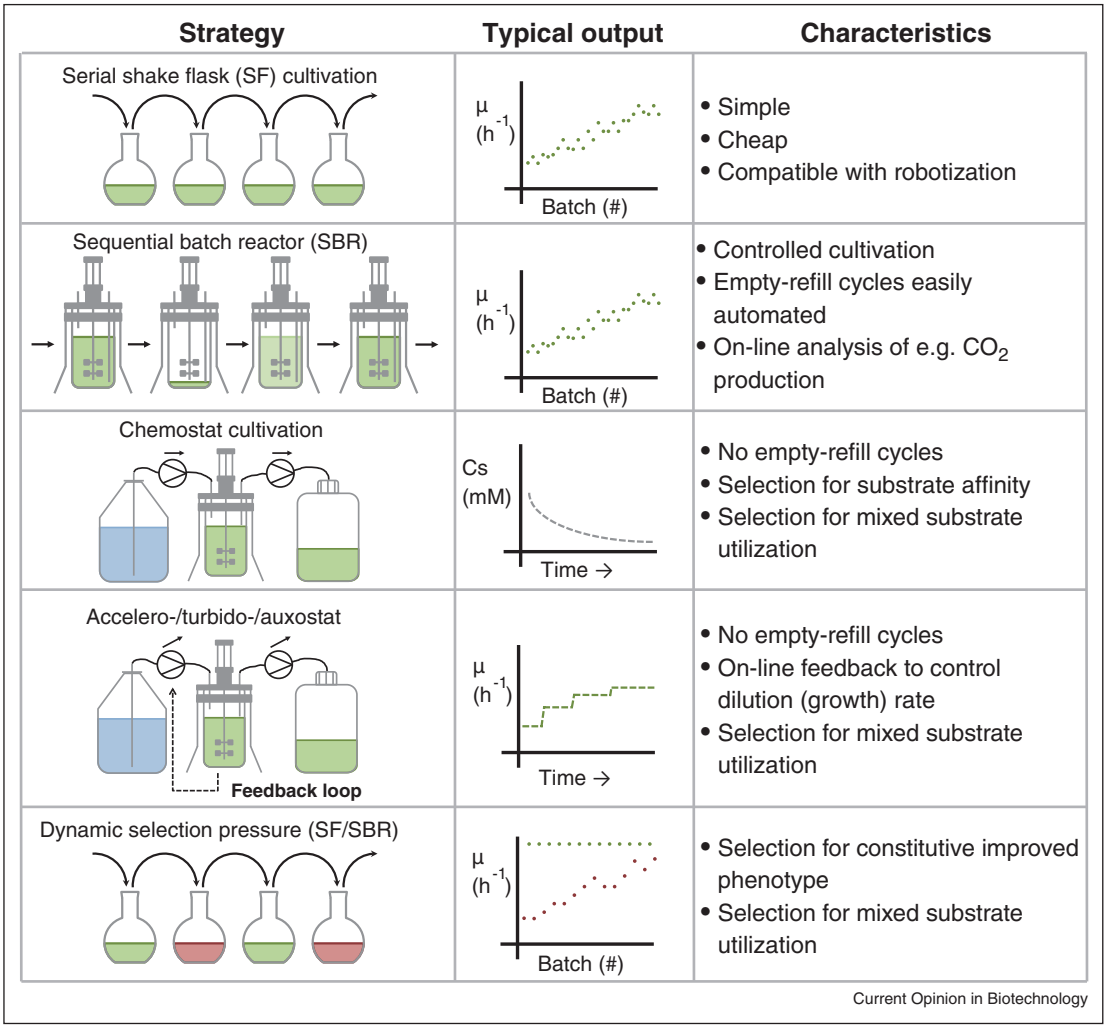
Selective pressure can be focused on a specific enzyme or cellular process by genetic engineering. For instance, deletion of all four genes encoding glucose-phosphorylating enzymes in pentose-fermenting *S. cerevisiae* strains enabled *in vivo* evolution of hexose-transporter variants that efficiently transported D-xylose and L-arabinose, two key sugars in lignocellulosic hydrolysates, in the presence of glucose (Table 1) [24•,25,26].

Identifying causal mutations: genome sequencing and classical genetics

Availability of near-complete, high-quality and strain-specific reference genomes [27,28], accurate whole-genome resequencing technologies and bioinformatics platforms have transformed evolutionary engineering into a powerful approach for understanding the genetic basis of complex, industrially relevant traits [29,30]. Moreover, CRISPR/Cas9-mediated, simultaneous introduction of multiple different targeted mutations [31] strongly accelerates functional analysis of the identified mutations via their introduction into non-evolved strains (Figure 2).

In addition to single-nucleotide mutations, insertions and deletions, evolved yeast strains frequently harbour whole-chromosome or segmental aneuploidies [32–34]. Particularly in the latter cases, only few of the affected genes may contribute to the phenotype of interest. Analysing multiple evolution experiments can help to identify causal mutations, especially when they affect the same genes or cellular processes in parallel experiments (Figure 2) [29]. For example, 3-hydroxypropionate-tolerant *S. cerevisiae* strains, isolated from three independent repeated-batch cultures, carried different mutations in

Figure 1



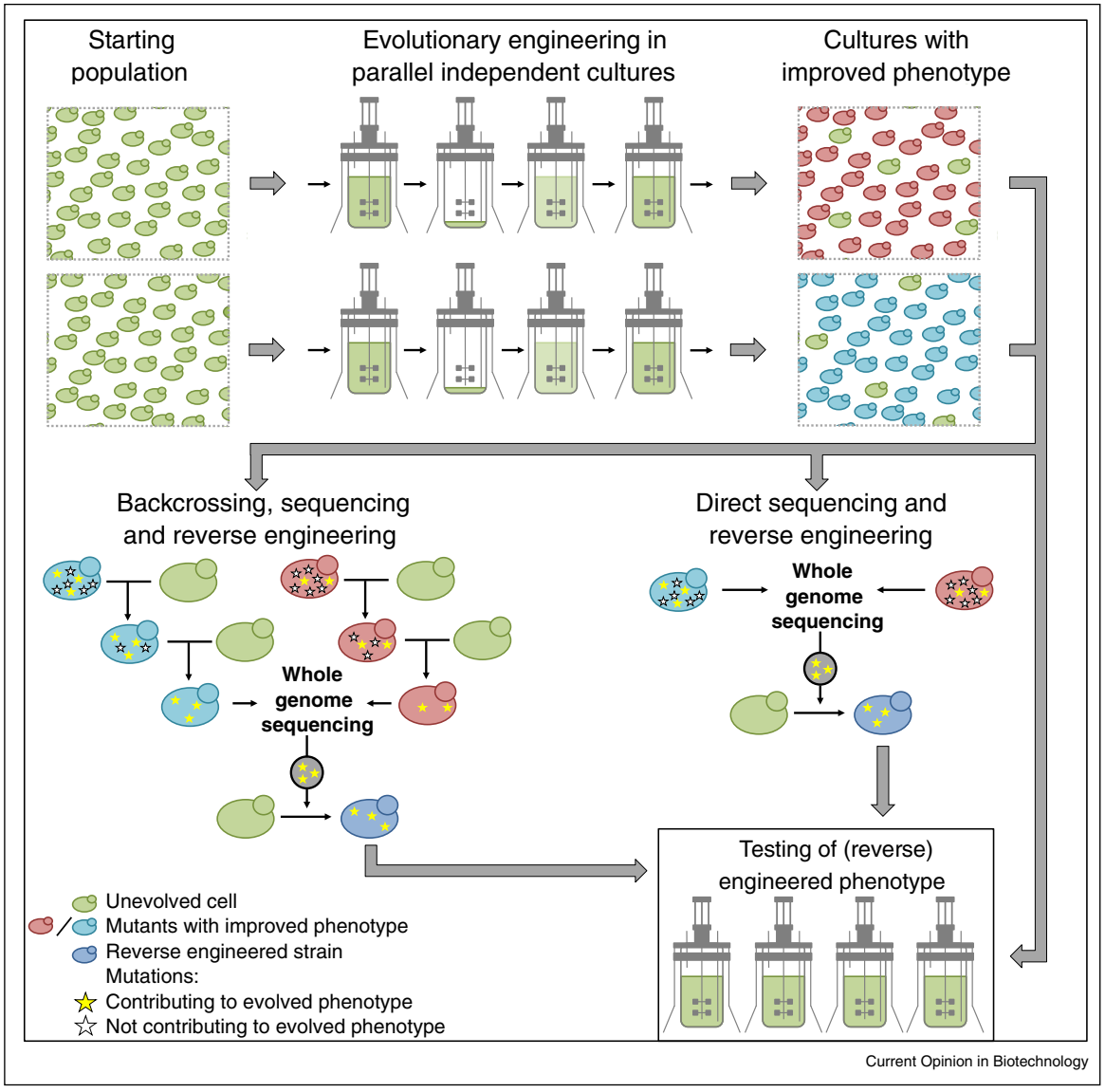
Overview of cultivation strategies used for evolutionary engineering. Red and green colors reflect cultures grown under different selective pressures (e.g. presence and absence of an inhibiting compound or growth on different substrates). The middle column illustrates typical development of specific growth rate (μ) or residual nutrient concentration (for chemostat cultivation, C_s) in evolving yeast cultures.

the S-(hydroxymethyl)-glutathione dehydrogenase gene *SFA1*. This observation enabled elucidation of a glutathione-dependent mechanism for 3-HP tolerance (Table 1) [35•].

Classical yeast genetics can accelerate identification of causal mutations (Figure 2). Back-crossing of evolved haploid strains with a non-evolved strain and subsequent tracking of mutated alleles in segregants that expressed the selected trait, enabled identification of mutations contributing to acetic-acid tolerance, butanol tolerance and fast glycerol utilization in evolved *S. cerevisiae* strains (Table 1) [36,37•,38]. When evolved genotypes are complex, for example, as a result of prolonged growth under conditions that increase mutation frequency, quantitative trait loci (QTL) analysis [39] is a powerful approach, as illustrated by a genetic analysis of improved ethanol tolerance in *S. cerevisiae* strains obtained in a 2-year evolutionary engineering campaign (Table 1) [40].

End-point analyses of evolution experiments do not necessarily capture all beneficial mutations that occurred during evolution and analysis of evolving populations can provide valuable additional information [41,42]. For instance, in a 450-generation experiment that selected for enhanced tolerance towards a mixture of inhibitors occurring in lignocellulosic hydrolysates, strains isolated at early time points showed a markedly higher tolerance to some of the individual inhibitors than the final evolved population [41].

Figure 2



Identification of causal mutations after evolutionary engineering of yeast strains. In haploid evolved strains, causal mutations can first be enriched by repeated backcrossing of evolved strains with a non-evolved strain of the opposite mating type (left). Alternatively, direct genome sequencing of strains originating from parallel evolution experiments can identify mutations that affect the same genes or processes in independently evolved strains (right).

Trade-offs and context dependency: benefits of dynamic selection regimes

Evolutionary engineering often reveals trade-offs between a selected trait and other aspects of yeast physiology. Trade-offs have been intensively studied in evolutionary engineering of *S. cerevisiae* for high-temperature tolerance, an important characteristic for bioethanol production. After serial transfer at supra-optimal temperatures, *erg3* null mutations were shown to strongly contribute to improved growth at 40 °C by causing replacement of ergosterol, the major sterol in wild-type membranes, by fecosterol (Table 1) [43]. Initial characterization demonstrated respiratory deficiency of the evolved thermotolerant strains [43], while further analysis revealed a reduced growth rate at 30 °C and increased glycerol production [44•,45].

Selection on single substrates favours mutants that preferentially allocate cellular resources to processes that directly contribute to growth on that substrate [46••]. Such a preferential resource allocation can go at the expense of the expression of proteins involved in other pathways [47]. Indeed, yeast strains evolved for improved growth on either D-xylose or L-arabinose often consumed

glucose and/or the other pentose sugar at reduced rates [48–51]. This trade-off was addressed by alternatingly growing an engineered, pentose-fermenting *S. cerevisiae* strain on different mixtures of glucose, xylose and/or arabinose in SBRs. By balancing the number of generations of growth on each of the three sugars, this dynamic selection regime yielded a strain that rapidly fermented sugar mixtures [49]. Prolonged nutrient-limited growth at low specific growth rates has been reported to result in reduced performance when the nutrient limitation was relieved (Table 1) [20,52,53]. Also in this case, dynamic selection regimes, for example based on alternating SBR and chemostat cultivation cycles, can prevent extreme trade-offs [22,54].

Yeast strains evolved for stress tolerance do not always express the acquired phenotype when the selective pressure is alleviated. Increased acetic-acid tolerance acquired after prolonged anaerobic continuous cultivation of a xylose-fermenting *S. cerevisiae* strain on acetic acid-containing medium, was not expressed in acetate-free medium [17]. Such an inducible tolerance is not compatible with industrial processes that involve a yeast propagation phase on acetic-acid-free media prior to conversion of acetic acid-containing lignocellulosic hydrolysates [3]. A dynamic serial-transfer strategy (Figure 1), in which cultivation cycles in acetic-acid-containing medium were alternated with cycles on acetic-acid-free medium, yielded strains with increased, constitutive acetic-acid tolerance [37•].

Accelerating evolution of yeast cultures

While chemical mutagens and radiation have long been used to increase mutation rates in microbes, genetic engineering offers new options to increase mutation frequencies in evolving cultures. Mutator yeast strains that increase mutation frequencies in a genome-wide manner can stimulate specific types of mutations. For example, *S. cerevisiae msh2Δ* strains exhibited a circa 40-fold increased frequency of single-nucleotide mutations and indels, while a *mec1Δ tel1Δ* genotype specifically stimulated large structural variations and chromosomal aneuploidy [55•]. When target sites of a heterologous recombinase are introduced at multiple genomic loci, expression of the associated recombinase causes deletions, inversions, duplications and more complex chromosomal rearrangements [56]. The Sc2.0 project, which designs and constructs synthetic *S. cerevisiae* genomes, exploits this feature by introducing hundreds of loxP sites. In the resulting strains, 'genome scrambling' induced by expression of the Cre recombinase can be used to generate genetic diversity in screening and evolutionary engineering experiments [57–59].

To increase mutation rates at a specific locus, the DNA glycosylase Mag1, which functions in base-excision repair, was fused to the Tet repressor, thus allowing precise targeting of the glycosylase to the 19-bp tet operator (tetO) sequence. Indeed, integration of a 240-copy tetO array efficiently recruited Mag1 to the target locus. Mag1-mediated base excision and subsequent repair by the error-prone DNA polymerase ζ, caused over 800-fold higher mutation rates in a 20-kb region surrounding the tetO array [60•]. In a study on retrotransposon-mediated, targeted mutagenesis [61] heterologous DNA was integrated between *Ty1RT* and the 3′-LTR of a galactose-inducible Ty1 retrotransposon in *S. cerevisiae*. Galactose induction resulted in mRNA formation, error-prone reverse transcription and reintegration of (mutated) cDNA, causing mutation rates of ca. 0.15 kb^{-1} per induction cycle in the target sequence and generating nearly 20 million distinct mutants per litre of culture. This method was successfully employed for *in vivo* mutagenesis of the *S. cerevisiae* global transcriptional regulator gene *SPT15* in an evolutionary engineering study on improving 1-butanol tolerance [61]. A very precisely localized increase in mutation rate was recently achieved by fusing a nuclease-deficient Cas9 (dCas9) to an activation-induced cytidine deaminase (AID). Expression of this complex in the presence of a guide RNA resulted in an increased occurrence of C to T mutations in a range of 3–5 bases in the dCas9 target site [62].

The incidence of copy-number variations of specific sequences can be enhanced by different techniques. When, during evolutionary engineering for fast growth on xylose, an expression cassette for a heterologous xylose isomerase (*XylA*) was integrated close to an ARS sequence, extrachromosomal circular DNA elements (eccDNA) carrying *XylA* were formed. These eccDNAs facilitated multi-copy chromosomal integration of *XylA*, after which the unstably replicating eccDNA was lost [63]. Integration of relevant genes in close proximity to eccDNA-forming ARS sequences, which frequently occur in the *S. cerevisiae* genome [64], offers an interesting approach for evolutionary 'tuning' of expression levels of relevant genes in engineered strains. Copy-number variation of relevant genes can also be facilitated by their integration between repetitive DNA sequences such as retrotransposons, as copy-number variation rates at such sites can be up to 5 orders of magnitude higher than elsewhere in the yeast genome [65,66]. Alternatively, tandem integration of multiple expression cassettes enables rapid copy-number expansion or compression by homologous recombination [67].

A holy grail in evolutionary engineering: improving anabolic product formation

Design of evolutionary engineering strategies that enable selection for traits that do not confer a selective advantage in wild-type genetic contexts represents key conceptual challenges. In particular, it would be highly interesting and relevant to harness evolutionary engineering for improving productivities and/or yields of 'anabolic'

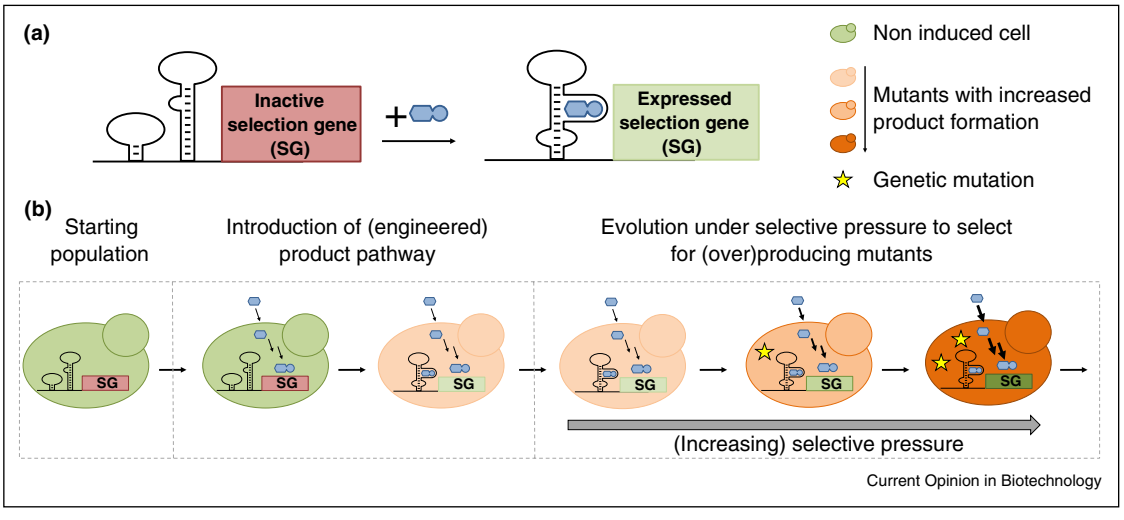
products, whose synthesis requires a net input of metabolic energy.

Some anabolic products have specific properties that can be used for designing selective growth regimes. For example, antioxidant properties of carotenoids have been exploited by evolving carotenoid-producing, engineered *S. cerevisiae* under hydrogen-peroxide stress [68] and increased buoyancy has been elegantly used to select for lipid-hyperaccumulating *Yarrowia lipolytica* mutants [69]. Other studies have sought to stoichometrically couple anabolic product formation to essential metabolic processes by genetic engineering and, thereby, enable growth-based selection regimes. In an early study, pathways for mitochondrial oxidation of cytosolic NADH were eliminated in a triose-phosphate-isomerase negative *S. cerevisiae* strain, thus leaving glycerol production as sole mechanism for NADH reoxidation. Serial transfer of the resulting strain at increasing glucose concentrations enabled isolation of a strain that accumulated over 200 g L^{-1} glycerol at a yield close to 1 mol (mol glucose)$^{-1}$ [70]. To couple production of succinate to growth, Otero *et al.* constructed a *S. cerevisiae ser3Δ ser33Δ sdh3Δ* strain, in which isocitrate lyase was essential for glycine and serine biosynthesis [71]. While subsequent evolutionary engineering improved succinate yields, stoichiometric coupling of product formation and growth was confined to a limited range of succinate yields.

Recent developments in research on synthetic regulatory circuits may enable the development of generically applicable strategies for evolutionary engineering of anabolic product formation. Sensor proteins that, upon binding of a compound of interest, activate expression of a fluorescent protein are already intensively applied for fluorescence-activated cell sorting of high-producing mutants [72]. New sensor systems continue to be developed for relevant compounds, as exemplified by the recent construction of two dose-dependent 1-butanol responsive promoters and their application for quantifying 1-butanol production by engineered *S. cerevisiae* strains [73]. In principle, synthetic sensor/promoter systems, for example based on product-specific riboswitches [74], could also be used to tightly couple product formation to an essential cellular process (Figure 3) [75,76]. In a pioneering yeast evolutionary engineering study, an aromatic amino-acid (AAA)-responsive hybrid promoter was used to control expression of the KanNeo gene, which confers weak resistance to geneticin. Serial transfer at increasing concentrations of geneticin and 4-fluorophenylalanine, an anti-metabolite of aromatic amino acid (AAA) synthesis, combined with random mutagenesis, yielded strains with a deregulated AAA pathway, which were used to improve precursor supply for muconic acid production (Table 1) [77••].

While use of synthetic regulatory circuits holds great promise for evolutionary engineering of anabolic product

Figure 3



Application of synthetic regulatory circuits in yeast evolutionary engineering, as schematically illustrated by the use of a product-responsive riboswitch. **(a)** Binding of the target product to the riboswitch causes dose-dependent expression (indicated by the darker shades of green in **b**) of a selection gene (SG), which encodes a protein whose intracellular level controls specific growth rate under selective conditions. **(b)** In engineered yeasts that co-express the target product pathway and the synthetic regulatory circuit, spontaneous mutants that produce higher levels of the target product (indicated by the darker shades of orange) exhibit a higher specific growth rate and thus can be selected for in evolutionary engineering experiments.

formation, several design criteria remain to be further investigated. To prevent selection of mutants that escape selective pressure by bypassing or 'killing' the regulatory circuit, introduction of multiple, redundant regulatory circuits is likely to be required. For instance, expression of multiple essential genes may be coupled to multiple, independent product-sensor/promoter combinations [78] and the dynamic range of the regulatory circuits should match or be easily adaptable to industrially relevant intracellular and/or extracellular concentrations of products or intermediates of interest. Designing, building and testing such strategies provides an industrially relevant scientific challenge at the interface of synthetic biology, microbial physiology and evolution biology.

Conclusions

Rapid developments in sequencing, analysis and editing of yeast genomes have transformed evolutionary engineering from a simple 'black box' strain-improvement strategy into an invaluable asset for understanding and rationally engineering yeast cell factories. Recent studies demonstrate how genetic engineering can confer a selective advantage to yeast strains with specific, industrially relevant phenotypes. Integration of carefully designed (dynamic) cultivation regimes, based on insight in yeast physiology and ecology, with methods for accelerating *in vivo* mutation rates at specific loci or across the yeast genome will further increase the impact of evolutionary engineering on yeast biotechnology. While still in its infancy, implementation of product-responsive, growth-coupled synthetic regulatory loops has the potential to addressing the longstanding challenge of harnessing the power of evolutionary engineering to enhance production of compounds whose synthesis by yeast cells does not confer a direct selective advantage. Designing, building and testing such circuits will involve exciting research at the interface of synthetic biology, yeast physiology and experimental evolution.

Conflicts of interest

None.

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- •• of outstanding interest

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