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Review

Opportunities for yeast metabolic engineering: Lessons from synthetic biology

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Constant progress in genetic engineering has given rise to a number of promising areas of research that facilitated the expansion of industrial biotechnology. The field of metabolic engineering, which utilizes genetic tools to manipulate microbial metabolism to enhance the production of compounds of interest, has had a particularly strong impact by providing new platforms for chemical production. Recent developments in synthetic biology promise to expand the metabolic engineering toolbox further by creating novel biological components for pathway design. The present review addresses some of the recent advances in synthetic biology and how these have the potential to affect metabolic engineering in the yeast *Saccharomyces cerevisiae*. While *S. cerevisiae* for years has been a robust industrial organism and the target of multiple metabolic engineering trials, its potential for synthetic biology has remained relatively unexplored and further research in this field could strongly contribute to industrial biotechnology. This review also addresses general considerations for pathway design, ranging from individual components to regulatory systems, overall pathway considerations and whole-organism engineering, with an emphasis on potential contributions of synthetic biology to these areas. Some examples of applications for yeast synthetic biology and metabolic engineering are also discussed.

Keywords: Industrial biotechnology · Metabolic engineering · *Saccharomyces cerevisiae* · Synthetic biology · Yeast

1 Introduction

The development of tools for the analysis and manipulation of gene expression over the past 30 years has affected industrial biotechnology, which aims to transform the way many chemical compounds are produced [1]. Metabolic engineering is generally defined as the manipulation of cellular properties to achieve for instance the enhanced production of a desired chemical compound and has played an important role in industrial biotechnology. The main appeal of metabolic engineering is that many chemical compounds of commercial interest can be produced via biological routes in ways that are more environmentally friendly, cost-effective and sustainable as compared to traditional methods. An especially promising area is the production of some naturally derived complex compounds. While such compounds have shown promise in medical applications, their extremely low natural abundance and complex synthetic routes make them infeasible for commercial production. Using metabolic engineering strategies, one could potentially engineer the natural producers of such compounds for enhanced production or, alternatively, transfer the synthetic pathways involved into a suitable industrial host that could then produce these compounds in high yields. Metabolic engineering in large part relies on advances in other fields (Fig. 1), such as developments in high-throughput analysis tools. The intro-

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Abbreviations: gTME, global transcriptional machinery engineering; **PTM**, post-translational modification; **rDNA**, ribosomal DNA; **UAA**, unnatural amino acid; **UTR**, untranslated region; **YAC**, yeast artificial chromosome; **YEp**, episomal plasmid; **YIp**, integrative plasmid

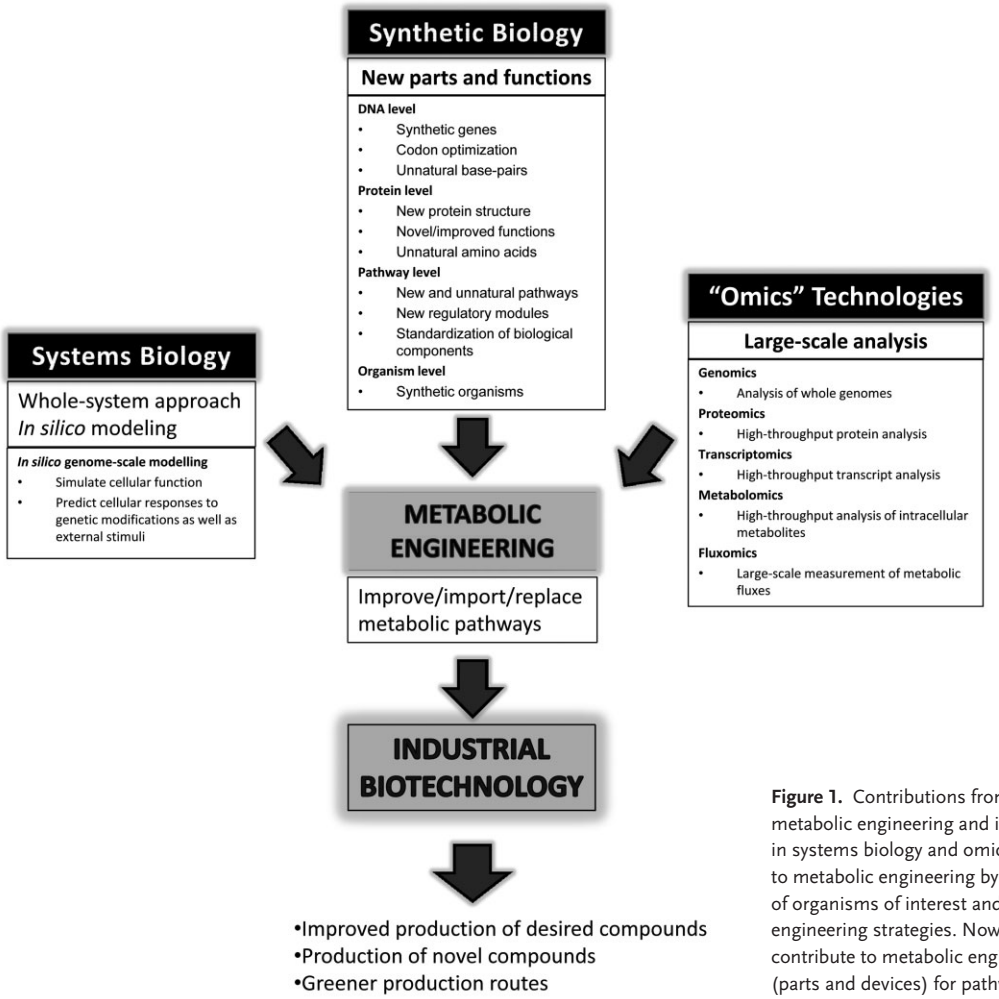

Figure 1. Contributions from advances in various fields to metabolic engineering and industrial biotechnology. Advances in systems biology and omics technologies could contribute to metabolic engineering by providing tools for global analysis of organisms of interest and prediction of optimal metabolic engineering strategies. Now, synthetic biology promises to contribute to metabolic engineering by creating new tools (parts and devices) for pathway design.

duction of "omics" technologies such as genomics, transcriptomics, proteomics and metabolomics [2–4] has made it easier to analyze the effects of genetic manipulation on cells, understand the mechanisms underlying beneficial phenotypes, and improve engineering strategies. In addition, systems biology and high-level modeling approaches such as genome-scale modeling contribute to metabolic engineering by characterizing cellular metabolism on a system level and predicting cellular responses to different metabolic engineering strategies [5–8].

Further contributions to metabolic engineering can come from the emerging field of synthetic biology. Synthetic biology involves the design and construction of biological parts and components that do not exist in nature and operates at several levels of biological organization. This includes the DNA level, through advances in gene synthesis and the use of codon optimization for optimal gene expression [9, 10], the creation of unnatural base pairs [11] and the use of quadruplet codons [12]. At the protein level, synthetic biology attempts to design new functions/specificities into proteins and create new UAAs (unnatural amino acids) that expand the catalytic possibilities further [13–14]. At the pathway level, synthetic biology attempts to redesign biological pathways, assemble completely new pathways from biological components or design novel regulatory systems [15, 16]. Even on the whole-organism level synthetic biology is making strides, with the first successful transfer of a synthetic genome reported recently [17]. In addition to creating new cellular molecules and functions, synthetic biology also attempts to standardize biological components, which could reduce the complexity associated with biological systems and make it easier to design, assemble and regulate metabolic pathways. Therefore, in many respects, synthetic biology complements metabolic engineering and advances in this field can create new and exciting tools for the engineering of microorganisms.

The complexity of eukaryotic systems can be viewed as both a challenge and an opportunity for synthetic biologists. For example, the presence of introns in eukaryotic genes further complicates gene expression, since these can have a significant effect on both the timing of gene expression [18, 19], as well as final protein structure through alternative splicing [20]. In addition, introns can harbor genes that code for small interfering RNAs, which can interfere with gene expression. The presence of different organelles in eukaryotes and the localization of proteins to different compartments add additional complexity that must be dealt with when attempting to engineer a metabolic pathway. Therefore, the innate complexity of biological systems is further exacerbated in eukaryotes and makes synthetic devices harder to engineer. However, this complexity also broadens the available toolset for synthetic biology [21] and opens up new avenues for synthetic biologists interested in engineering novel and sophisticated devices.

The yeast *Saccharomyces cerevisiae* makes for a particularly attractive production platform for industrial biotechnology. It is an extremely well-characterized organism, genetically, biochemically and physiologically. It was the first eukaryotic organism whose genome was sequenced [22] and several detailed online databases exist with relevant information about its genome and different omics data. In addition, *S. cerevisiae* is very tractable to genetic engineering, and many tools exist for its genetic manipulation. It is also a GRAS (generally regarded as safe) microorganism that is very industrially robust and can generate product yields in the multigram range. Because of these reasons, metabolic engineering has been frequently applied to *S. cerevisiae* to achieve desired properties (see [23] for a comprehensive review). However, despite the obvious benefits synthetic biology could bring to yeast metabolic engineering, efforts in this field have primarily focused on prokaryotic systems while yeast synthetic biology has lagged behind. The goal of this review is to discuss some of the recent advances in synthetic biology and how these are/could be applicable for metabolic engineering of *S. cerevisiae*.

2 Pathway engineering

2.1 Considerations for pathway engineering: Gene expression in yeast

One of the promises of synthetic biology is to contribute to the metabolic engineering toolbox and offer new strategies for pathway design. The engineering of synthetic pathways in microbial hosts could take on different forms. In some instances, the host's natural pathways might be manipulated in order to direct fluxes towards a particular metabolite of interest. In other cases, a natural biosynthetic pathway from one organism might be re-assembled in a heterologous host more suitable for industrial applications. Multiple enzymes from different organisms might also be assembled into a pathway in a heterologous host to mirror natural pathways. Finally, the most challenging (and possibly the most promising) design strategy is de novo pathway design, which involves the engineering of pathways using various unrelated enzymes to create new and unnatural metabolic routes towards compounds of interest.

As previously mentioned, one of the most appealing aspects of *S. cerevisiae* for synthetic biology and metabolic engineering applications is the ease of its genetic manipulation (see Table 1 for a summary of some commonly used manipulation strategies). Genes can be readily inserted, deleted, replaced, overexpressed or underexpressed [24]. Past efforts in genetic engineering in yeast have been geared towards heterologous overexpression of particular proteins of interest. Therefore, the chief objective of these efforts has been to get the highest possible yield of that particular protein. In contrast, design on the pathway scale requires different considerations. Chiefly, the overall flux through the pathway is the most important issue in pathway engineering, rather than the expression of individual genes. Overexpression could be harmful for several reasons. First, it typically relies on the use of high-copy plasmids. The continuous maintenance and high-copy replication of YEps (episomal plasmids) could pose a significant metabolic burden on the cell [25]. In addition, transcription and translation are very energetically expensive and therefore high expression levels of several heterologous components could also pose a metabolic burden to the cell, as well as drain the cell from nucleotides and amino acids [25]. These considerations taken into account, it could be better to engineer the individual enzymes in a pathway for high enzyme activities, rather than rely on overexpression to obtain high activities. However, enzyme activities that are too high could drain the cell from cofactors and other metabolites, as well as lead to the accumulation of potentially toxic intermediates in the cell. Therefore, for optimal results, pathway engineers must be able to get a proper balance between the expression and activities of different components.

Another important issue for pathway design is the stability of the vector used for the introduction

Table 1. Some commonly used yeast genetic manipulation tools

Manipulation	Tools available	Comments
Extra-chromosomal gene overexpression	Yeast episomal plasmids (YEps)-extra-genomic	Highest expression, but not very stable and require continuous maintenance
Genomic integration Promoter replacement/mutation Gene-tagging Targeted mutation Deletion	Yeast integrative plasmids (YIps) Gene-targeting/homologous recombination	Use yeast's efficient homologous recombination mechanism to easily manipulate the genome
Expression of very large sequences and multiple genes	Yeast artificial chromosomes (YACs)	Low expression levels, but allow expression of large pathways containing numerous genes, as well as testing combinations of genes from different sources for optimal pathway design
Regulation of gene expression	Constitutive and inducible promoters of different strengths	Promoters of various strengths can be generated through mutagenesis. Can be used to fine-tune gene expression

of heterologous pathways into the cell. In yeast, YEps are commonly used to overexpress heterologous or native genes. These plasmids have an origin of replication derived from the endogenous yeast 2-μm plasmid, allowing autonomous replication. They have a high copy number and therefore allow high expression of the genes they are carrying. However, such plasmids are not very stable [26] and their use requires cultivation in selective media. This is not very feasible in industrial settings and it is therefore best to choose a vector that is both segregationally stable and has a low copy number in order to minimize the metabolic burden on the cells. Copy numbers should also be consistent from cell to cell to ensure consistent production. The optimal way to accomplish this might be to integrate the pathway into the genome, since the genome is the most stable expression platform. Such integration can be easily accomplished with yeast using YIps (integrative plasmids). These plasmids do not possess a yeast origin of replication and can therefore be maintained only after chromosomal integration at a specific locus. While they do not allow the high expression levels observed with YEps, insertion of the gene into the genome results in high insert stability and does not require the continuous use of selective media. If necessary, overexpression can still be achieved by targeting genes into repeating DNA elements, such as ribosomal DNA (rDNA). The rDNA clusters are present in 150 tandem repeats and strains engineered this way result in mitotically stable vectors that are found in high copy numbers [27]. Multicopy integrations can also be achieved by targeting to solo long terminal repeats such as δ and σ sequences [28, 29].

A significant advantage of *S. cerevisiae* over other expression systems is the effectiveness of gene targeting. DNA-double strand breaks in yeast are preferentially repaired by homologous recombination [30] and linear DNA molecules are perceived as broken DNA fragments that need repair. If the DNA fragment is flanked by sequences identical to a target site in the genome, this fragment will be integrated into the target site via homologous recombination [31]. In addition to the insertion of genes at specific loci, this technique can also be used to delete or disrupt genes, introduce mutations into genes and tag genes with GFP (green fluorescent protein) or ubiquitin. Gene targeting could also be used to alter the expression of native genes by replacing or mutating their promoters. It should be noted that when choosing to integrate genes into the genome, the location of the inserts in the genome should also be considered, since it can affect gene expression levels [32].

Yeast artificial chromosomes (YACs) are artificially constructed chromosomes that contain telomeric, centromeric and replication origin sequences. While these vectors have traditionally been used to clone large sequences of DNA for library purposes, the use of YACs might also hold several unique advantages for metabolic engineering purposes. This was recently demonstrated by Naesby et al. [33], who used YACs to assemble a flavonoid pathway in *S. cerevisiae*. Results from this study have shown that YACs can express very large (100–3000 kb) sequences of DNA without causing extraordinary stress to the cell, which could be beneficial for the construction of large pathways. In addition, it was demonstrated that genes in a pathway could be assembled randomly in a single pro-

cedure. This could be particularly advantageous since it allows the simultaneous testing of a number of gene combinations from several different sources in order to find the optimal combination. Another advantage is that such random assembly of a large number of genes in a pathway could lead to the production and discovery of new naturally-derived molecules of potential interest. Considering the growing speed and decreasing price in DNA synthesis and the ease of assembly of large DNA molecules in yeast (see below), this approach will probably gain more attention in the future.

2.2 Yeast promoters and pathway regulation

Another important consideration in pathway design is the inducibility of the pathway. While constitutive induction of pathway components could result in higher product yields, it might also be quite stressful for the cell. Therefore, it might be better to induce the pathway at a particular point of interest. The use of inducible pathways allows the activation of a pathway at specific points in the production cycle and can be ideal for high-value chemicals. In contrast, constitutive production of the compound of interest might be required in cases where the use of inducers is too expensive. Due to these concerns, the use of appropriate promoters is extremely important to yeast genetic engineering.

The easiest and most effective strategy to control the inducibility of a pathway is using the inducible promoters. However, care should be taken to induce all cells in a culture uniformly to get consistent product yields. Among strong constitutive promoters, the most commonly used for high-level gene expression in *S. cerevisiae* are p*TEF1* and p*ADH1* as well as promoters derived from various glycolytic pathway genes. The best-studied inducible promoters are the different p*GAL* promoters, which are repressed by glucose and induced by galactose. These promoters allow high levels of expression under induced conditions.

Since the expression of large and complex pathways often requires fine tuning and balancing of the expression levels of single enzymes, the range of available promoters needs extension beyond the commonly used standard yeast promoters. For this purpose, high-throughput analysis methods are needed to be able to characterize large promoter libraries. Besides common reporter proteins such as β-galactosidase or different fluorescent proteins, a secretory luciferase has been applied recently to screen a library of 500 yeast promoters [34]. Such high-throughput assays can be used for characterization of natural as well as synthetic promoters. Synthetic promoter libraries have been constructed through e.g. error-prone PCR [35]. Alternatively, known promoter elements can be used, shuffled and/or combined with entirely randomized sequences [36, 37]. An approach that will probably gain more attention in the future is the employment of thermodynamic models to predict expression from synthetic as well as genomic promoters [38].

2.3 Alternative regulatory elements: RNA parts and devices

Current research in synthetic biology is exploring additional, promoter-independent regulatory modules for control of gene expression. For example, the use of RNA devices to control gene expression is a promising area of research that has generated some recent attention (for reviews see [39, 40]). RNA molecules can regulate gene expression by various mechanisms, including antisense binding to mRNA, conformational changes that disrupt transcription/translation and ribozyme activity [41, 42]. Therefore, RNA parts could be used as a powerful tool to tune enzyme levels, regulate pathway fluxes and optimize product yields. Since RNA parts are encoded by DNA, they can be easily introduced into the genome. In addition, their structural flexibility, ability to bind ligands and enzymatic properties make them especially advantageous for metabolic engineering and synthetic biology applications [40]. Some RNA regulatory molecules are *cis*-acting regulators that are found within the UTRs (untranslated regions) of the transcripts coding for their target genes. In contrast, other regulatory RNAs are *trans*-acting, exerting their effects on separate target transcripts [43]. The ability to undergo conformational changes in response to various signals allows RNAs to act as sensors and transducers in pathways. For example, they can undergo conformational changes in response to changes in temperature [44] and as such, function as temperature sensors.

Some RNAs, termed RNA aptamers, can bind different molecules in high affinities and high specificities. Ligand-specific aptamers can be generated using SELEX (systematic evolution of ligands by exponential enrichment) [45]. The ability to bind ligands can be particularly useful in pathway design, since it could allow the RNA-dependent regulation of gene expression in response to specific metabolites or other chemical signals. Win and Smolke [46] developed various RNA devices in *S. cerevisiae* that use RNA aptamers to sense small molecules, transmitter sequences that transmit information through conformational change and ac-

tuators that can interfere with mRNA translation, depending on the state of the sensor and the transmitter.

Multiple RNA parts can be assembled together to create RNA devices. Riboswitches (Fig. 2A) are an example of natural regulatory RNA elements that play roles in gene expression [47–50]. They are RNA molecules that are naturally found in the 5′ UTR of some RNAs and can regulate gene expression in response to changing metabolite levels through different mechanisms, involving transcription termination, translation initiation and mRNA processing [48, 49]. They are composed of two RNA parts: an aptamer (ligand-binding) do-

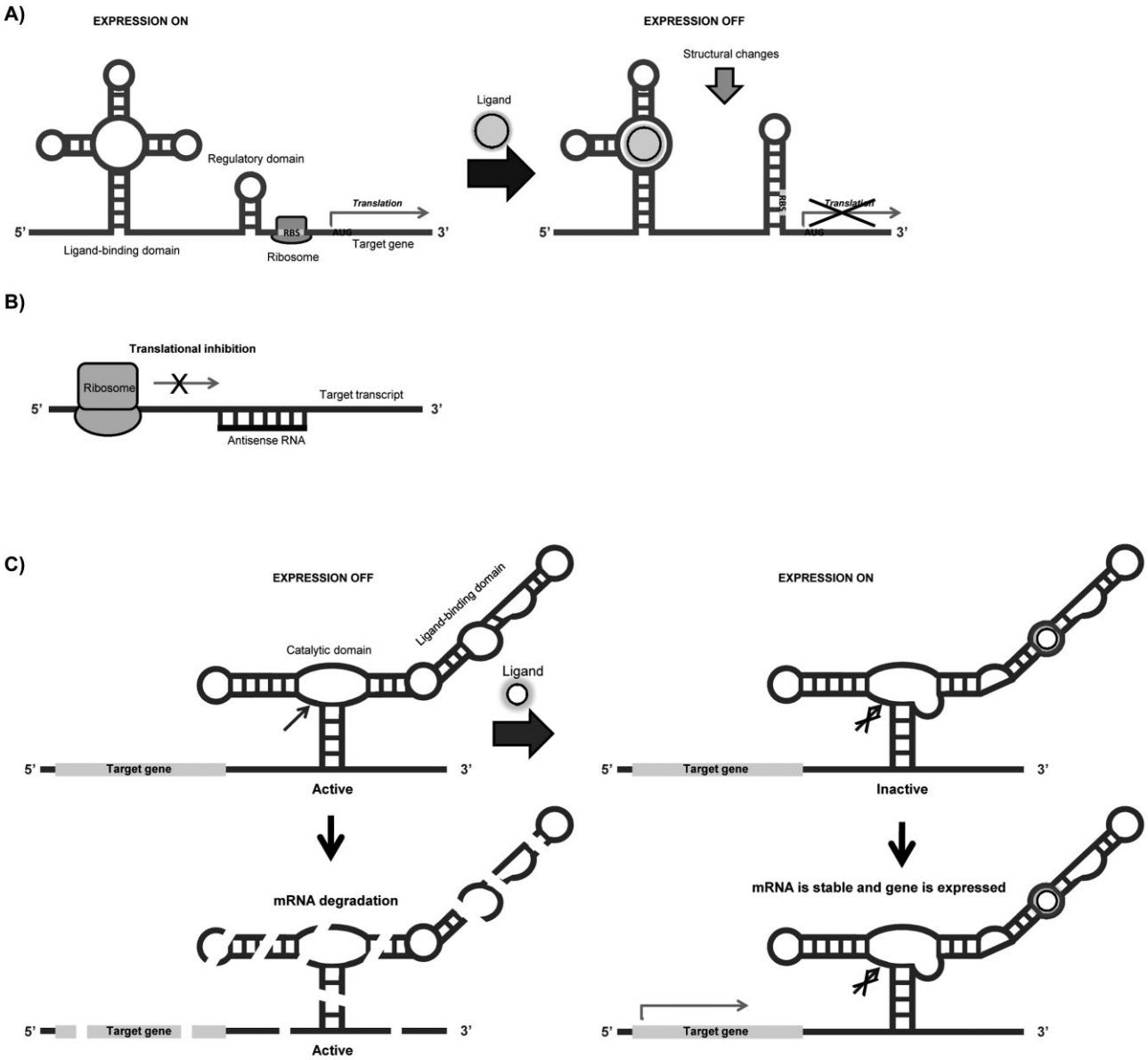

Figure 2. Some RNA devices used for regulation of gene expression. (A) An example for control of gene expression through riboswitch function. The riboswitch is composed of a ligand-binding aptamer domain and a gene-regulatory domain. Binding of a ligand to the aptamer domain facilitates structural changes in the riboswitch, resulting in changes in the expression of the downstream gene. In this example, these structural changes sequester the ribosomal binding site, resulting in inability to bind by the ribosome and translational inhibition. (B) Antisense RNAs. In this example, the antisense RNA binds to the target sequence and interferes with its translation, resulting in inhibition of gene expression. (C) Example of how ribozymes can be used to regulate gene expression in response to a ligand (adapted from [46]). In this example, the hammerhead ribozyme is fused to a ligand-binding aptamer domain and is embedded in a target transcript. In the absence of the ligand, the ribozyme is functional and the transcript is cleaved, resulting in inhibition of gene expression. In the presence of the ligand, binding to the ligand results in structural changes that render the ribozymes inactive and the target transcript is expressed.

main and a gene-regulatory domain. The aptamer domain can adopt a secondary and associated tertiary structure inside which the binding pocket for the target ligand is found. When the riboswitch binds a ligand, a conformational change is induced, resulting in a change in the expression of the downstream gene (reviewed in [50]). Therefore, riboswitches can be engineering into metabolic pathways for the promoter-independent regulation of specific pathway components. Such regulation systems could be especially useful for feedback mechanisms, where a riboswitch responsive for a particular pathway intermediate could down regulate upstream pathway components in response to accumulation of that intermediate until the intermediate is cleared. In *S. cerevisiae*, the use of artificial tetracycline-responsive riboswitches has been demonstrated [51–53]. A tetramethylrosamine-responsive switch that could activate transcription in a *trans*-acting matter was also engineered [54]. Tandem riboswitches or riboswitches with multiple ligand-binding domains can exhibit cooperative control over natural gene expression. This can be used to create complex circuits with devices that can control gene expression in response to different combinations of chemical inducers as recently demonstrated by Win and Smolke [55].

The antisense RNAs have also been used in synthetic biology to create regulatory devices (Fig. 2B). They are ssRNA molecules with a sequence complementary to the target transcript, allowing them to control the function of their target mRNAs through hybridization [56]. Such hybridization events can result in steric hindrance, preventing translation, or facilitate the cleavage of their target by dsRNA cleaving enzymes [57]. Antisense RNAs usually exert their functions by one of two mechanisms [58]. In the first mechanism, the hybridization event results in the targeting by RNA-cleaving enzymes. The second mechanism involves the binding of the antisense RNA around the translation start site of its target transcript, inhibiting translation. In *S. cerevisiae*, *trans*-acting antisense RNA parts complementary to the 5′ UTR of the target transcript have been used to silence the target [59]. In addition, Bayer and Smolke [60] used antisense RNAs to create complex devices by combining antisense and riboswitch properties to engineer "antiswitches" in *S. cerevisiae*. These RNAs regulated the ligand-responsive expression of target transcripts. The RNA interference (RNAi), a specific silencing pathway based on dsRNA, has been lost in *S. cerevisiae* during evolution. It was recently shown, however, that it could be restored by expressing two pathway proteins from a related budding yeast species [61]. Therefore, this mechanism could also potentially be used in the future to create new RNA devices in yeast.

Ribozymes represent another class of RNA parts that could be used to create regulatory devices for synthetic biology (Fig. 2C). Ribozymes are catalytic RNAs that catalyze the cleavage and/or ligation of RNA molecules [62–64]. Due to this ability, ribozymes could potentially be used to silence gene expression. The most commonly used ribozyme is the self-cleaving hammerhead ribozyme [65–67]. Win and Smolke [46] have attached hammerhead ribozymes to riboswitches, creating ligand-dependent ribozymes. These devices were embedded in the 3′ UTR of mRNA transcripts and the activation of the ribozyme resulted in mRNA cleavage, leading to decreased expression of the target transcript.

2.4 Protein engineering

The engineering of specialized parts is another area of interest for synthetic biology. Proteins represent the functional modules responsible for controlling pathway flux, and therefore engineering of protein properties is an important area of research. Continuing advances in this field promise to expand the list of biochemistries available for metabolic engineering and could potentially create new routes towards specific compound production. The activity of an enzyme in the cell is dependent on various traits such as its v_{max}, K_m, allosteric regulation, substrate/cofactor specificity, PTMs (post-translational modifications) and localization. Therefore, protein engineering efforts are often directed towards modifying these traits to achieve desired outcomes (for reviews of protein engineering see [68–71]).

The traditional methods in protein engineering involve the use of rational design, directed evolution or a combination of both. Rational design depends on extensive knowledge of protein structure and function to engineer new properties into proteins or enhance existing properties [72]. While this method is effective, the requirement for detailed knowledge regarding protein structure, function and catalytic activity is a major drawback, since this information is not available for most proteins. This method frequently involves sequence comparison of a protein family, which could give useful information on the amino acids that are potentially important for a specific function. These amino acids can then become the target of engineering efforts. In addition, when the protein structure is known, computer stimulations can be used to study the active site dynamics, substrate binding,

and folding properties of the enzyme. These modeling activities provide useful information for planning mutations. For example, molecular dynamics simulations have been used to identify flexible regions in proteins and subsequent introduction of disulfide bridges into these regions has resulted in a significant increase in protein stability [73]. Branduardi et al. [74] have also recently shown in *S. cerevisiae* that site-directed mutagenesis can be used to improve enzyme-specific activity using rational engineering approaches.

While rational design is very useful for protein engineering when detailed information regarding the protein is known, such information in unavailable for most proteins. Therefore, a significant number of protein engineering strategies rely on directed evolution, for which only knowledge of the protein sequence is required. This technique involves random mutagenesis of the protein structure, followed by high-throughput screening with the goal of evolving novel or enhanced properties into the protein. This usually involves the creation of a library of mutants of the gene of interest that is then tested against specific selection criteria (the property of interest). The best variants are then subjected to a new round of mutations. The diversity in such libraries is usually produced by random point mutations, recombination of genes, or a combination of both [75–77]. Because of their reliance on high-throughput screening or selection methods, a major drawback to protein evolution strategies is the availability of such screens since many proteins of interest to synthetic biology/metabolic engineering efforts might not have appropriate screens available. Advances in synthetic biology could be advantageous in this regard since they could allow the development of new gene networks that support high-throughput screens and selections. In such cases, the desired protein property (such as high enzyme activity) could be coupled with an easily identifiable trait (such as survival on a particular medium) that could then be used for high-throughput screening or selection.

A promising, but not well-explored area of protein engineering is the engineering of PTMs. Such modifications can affect enzyme activity, stability, localization and association with other molecules, and are therefore of key importance to controlling flux through pathways. A potential strategy of altering PTMs in proteins is by mutating specific residues, thereby preventing modifications on these residues. An example of this in yeast was demonstrated by Omura et al. [78], who replaced lysine residues in the cytoplasmic N-terminal domain of a Put4 permease with arginine. This decreased the ubiquitination of this enzyme, leading to a large increase in its levels independently of costly transcription and translation. Similar strategies could also be applied to residues that undergo other modifications to alter protein properties.

Recent advances in synthetic biology offer to open new avenues with respect to protein engineering and create new strategies aimed at enhancing the functions of existing proteins as well as create novel enzyme biochemistries. Research in this area has been largely directed towards the expansion of the genetic code to use new UAAs in proteins [14, 79, 80]. While the use of the 20 natural amino acids in proteins has created an enormous diversity of specificities and chemistries, expansion of the genetic code and the addition of UAAs promise to expand this further still, creating entirely new protein properties [81]. This in turn could significantly widen the spectrum of compounds that could be biologically produced and make a strong impact on industrial biotechnology. Because of the wealth of knowledge regarding its genetics and the ease of its genetic manipulation, *S. cerevisiae* was the first eukaryotic organism in which expansion of the genetic code to incorporate UAAs was attempted [80]. While this field of research is still in its infancy and a number of roadblocks must be overcome for industrial applicability, it shows a lot of promise. For example, studies have already shown that incorporation of UAAs into proteins has resulted in improved traits, such as improved stability [82, 83] and improved activity [84, 85].

While traditional protein-engineering methods can be used to eliminate PTMs from a protein (as described above); UAAs can be used to engineer permanent modifications into proteins. For example, a phosphorylation state that is resistant to the action of phosphatases was engineered into proteins in *Escherichia coli* [86]. This could be particularly useful when attempting to transfer a pathway from one organism into an unrelated host that might not naturally posses the machinery required to post-translationally modify proteins for optimal function.

2.5 Synthetic regulatory networks

The approaches above aim mainly at modulating single pathway components. However, the future of metabolic engineering will most likely lie not only in the assembly of novel pathways, but also in providing these pathways with regulatory systems e.g. in order to maximize product formation while minimizing the metabolic burden on the host organism. Artificial regulatory circuits designed for this purpose could either function orthogonally to the host

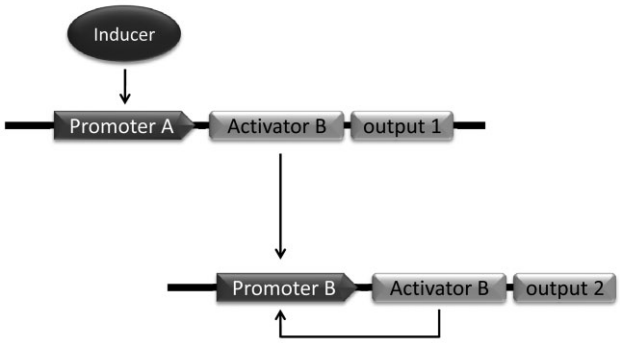

Figure 3. Using a positive feedback loop to create a memory device in yeast (adapted from [87]). In response to an inducer, promoter A becomes activated, leading to increased expression of activator B and output 1. Activator B acts on promoter B, leading to increased expression of the same activator.

cell's biology, which would ease their design and predictability, or they could interact with the already existing network, which would for instance enable coupling product formation with a certain cellular state. In order to create such circuits, existing and characterized modular parts such as promoters, activator/repressor-binding sites, regulatory RNAs or protein domains are logically recombined and their behavior is described by mathematical models (reviewed in [87, 88]). In the past decade, several artificial regulatory circuits have been constructed, mainly as proof-of-principle studies in *E. coli*. *S. cerevisiae*, in which behavior like transcription factor binding can be more difficult to predict than in prokaryotes [38], has also been employed in some of these studies. Autoregulatory positive feedback loops have been used to create bistable systems [89] or systems that can even enter a memory state ([90] and Fig. 3). Synthetic circuits were used to control the timing of yeast flocculation [37] or to create an aging yeast population by inhibiting growth of daughter cells [91]. However, several challenges such as increasing the library of well-characterized modular parts for circuit design and improving their predictability still need to be addressed before these systems can become routine application in metabolic engineering [92].

3 Engineering on the organism level

Microbes can be further manipulated at the cellular level. This is commonly termed "evolutionary engineering" and involves various methods used for strain improvement [93]. A common approach to evolutionary engineering involves many cycles of genetic modifications (commonly mutagenesis) followed by selection, resulting in new strains with desirable traits. While this field does not strictly fall under the definition of metabolic engineering or synthetic biology, it can often be the next logical step to both (i.e. once a new pathway has been engineered into a cell, evolutionary engineering could be used to further improve yield, or robustness of the resulting strain). Like other metabolic engineering approaches, evolutionary engineering has been greatly helped by "omics" technologies: advances in sequencing technologies have proven very useful for the characterization of mutant genomes, facilitating identification of specific mutations, while transcriptomics has been helpful in identifying specific gene expression changes [94]. Another evolutionary method explores natural adaptation of organisms to their environment [93]. Here, improved phenotypic traits are obtained by growing the organism under conditions of increasing selection pressure.

A related metabolic engineering approach is inverse metabolic engineering. It is defined as "the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; Secondly, determining the genetic or the particular environmental factors conferring that phenotype; and thirdly, endowing that phenotype on another strain or organism by direct genetic engineering or environmental manipulation" [95]. Inverse metabolic engineering is a natural follow-up to the evolutionary techniques mentioned above insofar that following the generation of strains with desirable properties, the genetic base of these properties could be identified and transferred into a production strain. In the case of *S. cerevisiae*, evolutionary engineering has been used to improve utilization of carbon sources [96–98], improve stress-tolerance [99] and achieve high product yields [100, 101].

Another recently developed technique for the creation of strains with beneficial phenotypes is gTME (global engineering of transcriptional machinery) [102, 103]. This involves the random mutagenesis of cellular transcriptional machinery followed by selection of organisms with desired traits. This technique has been shown to create increased diversity of phenotypes compared to classical mutagenesis techniques and increases the chance of finding beneficial mutants.

4 Applications for yeast synthetic biology and metabolic engineering

Metabolic engineering and synthetic biology strategies have already been applied to *S. cerevisiae*

to produce compounds of interest. Since this topic has been extensively reviewed elsewhere [23], only a few key examples will be mentioned here.

4.1 Production of isoprenoids

There is much interest in the microbial production of isoprenoids that are naturally produced by plants, since these can find use as perfumes, flavors and pharmaceuticals [104]. Isoprenoids are a large and structurally diverse group of plant metabolites, with more than 40 000 compounds discovered so far [105]. Often the production of plant isoprenoids in microorganisms requires the expression of P450 menooxygenase enzymes, which can be difficult in bacterial host systems. There is therefore much focus on metabolic engineering of *S. cerevisiae* towards isoprenoid production. Isoprenoids can be classified into several groups, such as mono-, sesqui-, di-, sester-, tri-, tetra- and polyterpenes, but the biosynthesis of all isoprenoids starts from isopentenyl diphosphate (IPP), an intermediate of the mevalonate pathway in yeast, while the universal precursor for monoterpenoids is geranyl diphosphate (GPP), which occurs in yeast as an intermediate of farnesyl pyrophosphate (FPP) synthesis. FPP is the precursor of sesquiterpenes.

Several metabolic engineering strategies have been used in recent years to produce isoprenoids of interest in *S. cerevisiae*. One approach attempted to increase the general production of isoprenoids in yeast by increasing the supply of acetyl-CoA. *S. cerevisiae* produces FPP through the mevalonate pathway using acetyl-CoA as the starting point, and an increase in its levels could contribute to increased isoprenoid production. In order to increase acetyl-CoA availability Shiba et al. [106] engineered the pyruvate dehydrogenase bypass in *S. cerevisiae*. The *S. cerevisiae ALD6* gene, encoding an acetaldehyde dehydrogenase was overexpressed, and a *Salmonella enterica* acetyl-CoA synthetase was introduced. This increased the carbon flux into the mevalonate pathway and resulted in increased amorphadiene production. In another study, the expression of geraniol synthetase from *Ocium basilicum* efficiently converted GPP to geraniol, a monoterpenoid [107].

With the objective of producing fragrances in yeast, Asadollahi et al. [108] introduced heterologous genes for the synthesis of valencene, cubebol and patchoulol. In order to enhance the availability of FPP, the *ERG9* gene (which is responsible for conversion of FPP to squalene) was down-regulated by expressing it under a methionine repressible promoter. This resulted in the accumulation of FPP-derived fragrance compounds, and further improvement in sesquiterpene production was achieved by adjusting the methionine level during fermentations. In another study, a genome-scale model was used to identify new targets for enhanced sesquiterpene biosynthesis in *S. cerevisiae* [109]. The NADPH-dependent glutamate dehydrogenase (encoded by *GDH1*) was identified as a potential candidate for deletion and its deletion was expected to enhance the availability of NADPH in the cytosol for the NADPH-requiring mevalonate pathway. Subsequent deletion of this gene led to an 85% increase in the final titer of cubebol, but also led to decrease in the specific growth rate. This was fixed by overexpressing the NADH-dependent glutamate dehydrogenase (encoded by *GDH2*). Additionally, the catalytic domain of hydroxymethylglutaryl-CoA reductase Hmg1 was overexpressed in *S. cerevisiae*, resulting in increased intracellular pools of FPP and a higher production of cubebol and squalene [110].

A well-known example of the use of metabolic engineering and synthetic biology strategies for the production of pharmaceuticals is the re-engineering of *S. cerevisiae* mevalonate pathway for the production of artemisinic acid. Artemisinin is a sesquiterpene lacton endoperoxide that is naturally found in *Artemisia annua* (sweet wormwood) and is known to be a highly effective drug against malaria. However, this compound is in short supply and therefore unavailable to most subjects suffering from this disease. While the total chemical synthesis of artemisinin is very difficult and costly, its semi-synthesis from artemisinic acid, its precursor, is a cost-effective and environmentally-friendly production strategy. Ro et al. [111] therefore engineered *S. cerevisiae* to produce artemisinic acid, resulting in high titers.

Taxol has been another target of metabolic engineering in *S. cerevisiae*. Taxol is a diterpenoid produced by secondary metabolism of yew trees (*Taxus sp.*). It is a widely used anticancer compound. However, only limited amounts of Taxol or related metabolites can currently be produced, and therefore, there is significant interest in producing high titers of this metabolite through metabolic engineering. Recently, Dejong et al. [112] have reconstructed a partial taxol biosynthetic pathway by expressing five sequential pathway steps. However, only the initial steps of the pathway appeared to be functional in the constructed strain, so that the intermediate taxadiene was accumulated.

Another example of a pharmaceutically interesting pathway is the one leading to formation of hydrocortisone. It was established in yeast by expressing one plant enzyme and eight mammalian proteins [113]. The pathway was partially directed

to the cytosol and partially to the mitochondria. Optimization strategies included the elimination of unwanted side reactions and balancing of gene expression by modulating copy number and promoter strength. One challenge in constructing such a complex pathway is the accumulation of intermediates or by-products that may have toxic effects on the host organism. Here, synthetic biology can provide the tools necessary to establish regulatory circuits in order to eliminate these side effects.

4.2 Polyketides

The production of polyketides, a group of natural compounds with medicinal properties represents another area of interest to metabolic engineers. Mutka et al. [114] introduced pathways for production of methylmalonyl-CoA, a precursor for complex polyketides into *S. cerevisiae*. In another study, the 6-methylsalicylic acid synthase (6-MSAS) gene, a polyketide synthase gene required for the synthesis of 6-methylsalicyclic acid (6-MSA) was heterologously expressed in *S. cerevisiae* [115]. A 4′-phosphopantetheinyl transferase (PPTase) gene required for the activation of 6-MSAS was co-expressed, resulting in high titers of 6-MSA. A further attempt to enhance 6-MSA production was made by enhancing the supply of precursors for 6-MSA production [116]. The promoter of the native acetyl-CoA carboxylase gene (*ACC1*), which encodes the enzyme for conversion of acetyl-CoA to malonyl-CoA, was replaced with a strong constitutive promoter in a strain carrying plasmids with genes encoding 6-MSAS from *Penicillium patulum* and a PPTase from *Aspergillus nidulans*. A 60% increase in the production of 6-MSA was observed compared to a strain having the native promoter in front of *ACC1*.

4.3 Butanol

The increased oil demand, diminishing oil resources and concerns over climate change have in recent years stimulated interest in the search for alternative fuel sources [117, 118] and the production of biofuels has been getting a lot of media coverage. Recently, butanols have been getting increasing attention as potential biofuels. Butanols (1-butanol, 2-butanol and iso-butanol) have sufficiently similar characteristics to gasoline to be blended directly into it. In addition, they are also superior to ethanol as a fuel due to their higher energy content and lower volatility, hygroscopicity and corrosiveness [119, 120]. The biological pathway for the production of 1-butanol exists in the *Clostridia* species of bacteria, and these organisms have been previously used for butanol production [121, 122]. However, they are extremely difficult to maintain, cultivate and genetically engineer. In addition, they can only be grown anaerobically and form large quantities of by-products, such as butyrate, acetone, and ethanol [123]. Therefore, there is a strong interest in the reconstruction of the butanol pathway in more commonly used industrial microorganisms. A synthetic strategy for the production of 1-butanol has recently been applied to *S. cerevisiae* [124]. The authors of this study reconstructed the butanol pathway and evaluated several different enzyme sources. While butanol was produced by the engineered strains, the highest titer obtained was 2.5 mg/L, which is very low in comparison with 20 g/L titers achievable with *Clostridia*. Therefore, further optimization of this pathway is required.

5 Yeast as a tool for genome assembly

While for the most part, this review has focused on potential applications of synthetic biology towards metabolic engineering purposes, it should also be mentioned that *S. cerevisiae* in itself could be used as a tool for synthetic biology in other organisms. It has been recently demonstrated that *S. cerevisiae* can be used in genome transplantation [125, 126]. This technique allows the modification of genomes from other organisms in yeast, followed by transplantation into the original cell. The chief benefit of this strategy is that it allows the full array of yeast genetic manipulation techniques to be applied to organisms that are not as genetically tractable, do not have well-developed genetic tools or are difficult to cultivate [127]. This technique can be used to get better information on these organisms, or to manipulate them for industrial purposes.

The most recent example in which genome transplantation in yeast has been applied to synthetic biology is through the assembly of the first synthetic genome [17]. This work was largely accomplished using *S. cerevisiae* as a tool for the assembly of the *Mycoplasma mycoides* genome. Various methods were developed to allow the transplantation of the synthetic genome out of yeast, including methods for cloning entire bacterial chromosomes as centromeric plasmids in yeast. The whole synthetic genome (582 970 bp) was therefore grown in yeast cells as a yeast centromeric plasmid. Maintenance of the genome in the yeast cell allowed the researchers to have a system in which they could test different optimization strategies. In addition, by amplifying the genome in yeast, the researchers had access to technologies such as gene

deletion/insertion and other manipulation techniques not normally available for the source organism.

6 Conclusions

Because of the large collection of tools available for its genetic manipulation, the ease of such manipulation and its well-established role as a robust industrial organism, the yeast *Saccharomyces cerevisiae* has been a common target for metabolic engineering. Such attempts were aimed at improving various natural traits or engineering completely new ones. Recent advances in synthetic biology promise to contribute to metabolic engineering of *S. cerevisiae* by creating new parts and devices. However, while *S. cerevisiae* seems like a natural candidate for eukaryotic synthetic biology, most synthetic biology studies to date have been primarily conducted in prokaryotic models. Expansion of synthetic biology to yeast promises to provide novel tools for metabolic engineering of this organism, and in turn strongly contribute to industrial biotechnology. This will call for building extensive libraries of promoters, codon optimized genes, terminators, integration sites, and expression vectors, each extensively characterized at different growth conditions, such that the synthetic biologist and/or metabolic engineer can directly choose from the library when new pathways are to be assembled or existing pathways are to be re-engineered. There is some progress in this [32, 128] but far more extensive libraries are needed before it will be possible to rapidly construct new and efficient cell factories.

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