FEMS Yeast Research, 15, 2015, 1–10

doi: 10.1111/1567-1364.12185
Minireview

MINIREVIEW

Recent applications of synthetic biology tools for yeast metabolic engineering

Michael K. Jensen[1,*] and Jay D. Keasling[1,2,3,4]

[1]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Hørsholm, Denmark, [2]Joint BioEnergy Institute, Emeryville, CA, USA, [3]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA and [4]Department of Chemical and Biomolecular Engineering & Department of Bioengineering University of California, Berkeley, CA, USA

*Corresponding author. The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kogle Allé 6 DK-2970 Hørsholm, Denmark, Tel.: +45 6128 4850; E-mail: mije@biosustain.dtu.dk
Editor. Hal Alper

ABSTRACT

The last 20 years of metabolic engineering has enabled bio-based production of fuels and chemicals from renewable carbon sources using cost-effective bioprocesses. Much of this work has been accomplished using engineered microorganisms that act as chemical factories. Although the time required to engineer microbial chemical factories has steadily decreased, improvement is still needed. Through the development of synthetic biology tools for key microbial hosts, it should be possible to further decrease the development times and improve the reliability of the resulting microorganism. Together with continuous decreases in price and improvements in DNA synthesis, assembly and sequencing, synthetic biology tools will rationalize time-consuming strain engineering, improve control of metabolic fluxes, and diversify screening assays for cellular metabolism. This review outlines some recently developed synthetic biology tools and their application to improve production of chemicals and fuels in yeast. Finally, we provide a perspective for the challenges that lie ahead.

Key words: synthetic biology; production; chemicals

INTRODUCTION

Yeast has been widely used for production of a variety of small and large molecules, including alcohols, acids, hydrocarbons, and proteins. Its fast growth on relatively cheap carbon sources, well-developed genetics, an endogenous metabolism capable of producing precursors to useful compounds, robustness in large-scale fermentations, and resistance to inhibitors and phages (Hong and Nielsen, 2012) have made it the preferred host for many bioprocesses (Buchholz and Collins, 2013). Also, the availability of genetic tools to perturb metabolic pathways, publicly available knockout collections, and the simple generation, isolation and analysis of yeast mutants make yeast an attractive host. Industrially, several companies use yeast to produce ethanol, butanol, farnesene, lactic acid, and artemisinin, to name a few. Despite its wide use, there are still many challenges in engineering yeast to improve titers, rates and yields of existing products and to produce novel products.

Synthetic biology aims to capitalize on nature's chemical and biological diversity by enabling the introduction of orthogonal, scalable, and robust functionalities into any living system and expands our ability to harness native biological systems for a wide range of applications (Nielsen *et al.*, 2013). Though still regarded as a young discipline, the tools developed by synthetic biologists have already facilitated metabolic engineering for bio-based production of fuels for transportation, health-promoting nutraceuticals, noninvasive diagnostic tools for specific pathogens, and active pharmaceutical ingredients (Shin *et al.*, 2012; Lan and Liao, 2013; Kotula *et al.*, 2014). The key-enabling technologies to these successes include the ever-decreasing cost in DNA sequencing and synthesis, the continuously improved knowledge on sequence-to-function

Received: 4 June 2014; **Accepted:** 10 July 2014

relationships of both DNA parts and proteins, standardization of DNA assembly procedures, and novel sensor-reporter systems.

This review focuses on the most recent advances in synthetic biology tools and how they have impacted yeast metabolic engineering. Although examples applying synthetic biology tools in nonconventional yeasts are emerging, most of the methods covered in this review address applications in *Saccharomyces cerevisiae*. Also, to limit the scope of this review, we will emphasize the most recently developed synthetic biology tools and their current applications for chemicals and fuels production. Finally, we will provide a perspective for what challenges the development of synthetic biology tools faces in the near future to advance metabolic engineering even further. For earlier reviews covering synthetic biology tools for advancing bio-based production, readers are referred to Siddiqui *et al.* (2012) and Borodina and Nielsen (2014).

GENE EXPRESSION TOOLS

Modular genetically encoded parts with assigned biological functions such as promoters, terminators, sensors, and reporters are platform synthetic biology tools to control, balance, and predict pathway fluxes through gene expression perturbations. This section serves as a general introduction to some of the most recently published gene expression tools applied to yeast for improving chemical and fuel production.

Transcription-based tools

Promoters and terminators

One of the most straightforward ways to manipulate protein levels inside the cell is to control the production and stability of mRNA. To design and engineer protein levels based on transcript production and stability, we need functional descriptors for promoters and terminators that not only describe their input–output characteristics under a variety of conditions (Blazeck *et al.*, 2012; Marchisio, 2014) but also how small changes in DNA sequence will impact their function. A few recent studies have sought to broaden our basic understanding of transcriptional regulation at the single-nucleotide level and improve the characterization of regulatory parts during multiple conditions relevant for bio-based production. For a broader overview on yeast promoter structure and applications using native and synthetic promoters, readers are referred to Hubmann *et al.* (2014).

To identify functional parts descriptors, Rajkumar *et al.* (2013) characterized more than 200 synthetic promoters using the *PHO5* promoter of *S. cerevisiae* as a platform part. This study identified strong relationships between *in vitro* identified transcription factor:promoter binding affinities and the quantitative output from the synthetic promoters. From these relationships, the authors were able to apply a simple binding model to explain more than 95% of the observed variance in their data. Such studies enable predictable tuning of *in vivo* promoter activity based on simple TF:DNA-binding affinity measurements (Rajkumar *et al.*, 2013). Also, Tyo *et al.* (2011) applied error-prone PCR to *TEF1* and *DAN1* promoters of *S. cerevisiae* and studied the characteristics for each promoter to allow for easy selection of the promoters needed for the specific genetic circuit described *in silico* (Tyo *et al.*, 2011). Alper *et al.* have also produced libraries of robust, tuneable and synthetic hybrid promoters harboring combinations of selected regulatory elements (REs) and core promoters, allowing enhanced tuneability compared to native *S. cerevisiae* and *Yarrowia lipolytica* promoters and, most importantly, defined control of promoter activity (Fig. 1a) (Blazeck *et al.*, 2012, 2013). In addition to promoters, combinatorial assemblies of terminators and promoters have identified both stabilizing and degradation-prone terminators that impact mRNA stability by almost two orders of magnitude (Curran *et al.*, 2013; Yamanishi *et al.*, 2013).

For synthetic biology to be even further applicable to large-scale fermentation settings and feedstock composition used in bio-based production, emphasis should be placed on characterizing parts under such industrial bioprocess conditions (Moser *et al.*, 2012). To gain a deeper understanding of the impact of environmental conditions on expression from yeast promoters, Partow *et al.* (2010) reported varying activities for seven constitutive and glucose-based promoters, under various glucose concentrations and fed-batch vs. continuous cultivation. Similarly, for terminators, Ito *et al.* (2013) investigated whether the activity of five strong terminators was affected by carbon source utilization, stress factors, yeast strain, or stage of the growth phase (Ito *et al.*, 2013). Interestingly, terminators were found to display conditional activities as also observed for promoters. Likewise, the ongoing development and interrogations of condition-dependent regulation of metabolism in yeast offer dynamic insights to reactions that change conditionally both in flux and transcription (Österlund *et al.*, 2013). Such knowledge is of paramount importance for synthetic biology to offer controlled timing (i.e. growth vs. production, before vs. after diauxic shifts) and improved predictability under industrial bioprocessing conditions.

Indeed, the conditional promoter activity of the gene-encoding squalene synthase (*ERG9*) was recently tweaked for re-directing the key mevalonate pathway intermediate farnesyl diphosphate (FPP) from sterol synthesis to production of sesquiterpenes (Paradise *et al.*, 2008; Scalcinati *et al.*, 2012). Using an intelligent dynamic control of *ERG9* expression, in which Scalcinati *et al.* (2012) swapped the *ERG9* promoter with the glucose-responsive *HXT1* promoter, α-santalene levels were improved by 3.4-fold compared to native *ERG9* expression (Table 1). In addition to *ERG9*, tuning expression of upper mevalonate pathway enzymes and P450 cytochromes and their reductases was applied for the engineering of artemisinic acid production in yeast, thereby reaching titers of more than 40 g L^{-1} for the artemisinin precursor amorphadiene, and 25 g L^{-1} of artemisinic acid (Westfall *et al.*, 2012; Paddon *et al.*, 2013). Artemisinin is the key ingredient in artemisinin-based combination therapies to combat malaria, and the further development of a chemical process for conversion of artemisinic acid to artemisinin has formed the basis of a viable industrial process for the production of semi-synthetic artemisinin (Paddon and Keasling, 2014).

In addition to controlling the expression of key intermediate steps, interrogating the effect of changes in expression levels of individual genes in multistep production pathways has been sought to alleviate flux imbalances known to compromise production performance. Using a combinatorial approach to identify optimal flux through a multistep pathway using several, different, characterized promoters, two recent studies screened for optimal violacein and oleochemical production in *S. cerevisiae* and *Y. lipolytica*, respectively (Lee *et al.*, 2013; Blazeck *et al.*, 2014a,b). From such an approach, Lee *et al.* (2013) were able to train a regression model from a random subpopulation of violacein-producing strains and then use this model to predict ($0.84 < r < 0.92$) genotypes that would preferentially produce each of the products (violacein, deoxyviolacein, proviolacein, and prodeoxyviolacein) in the branched violacein biosynthesis pathway (Lee *et al.*, 2013).

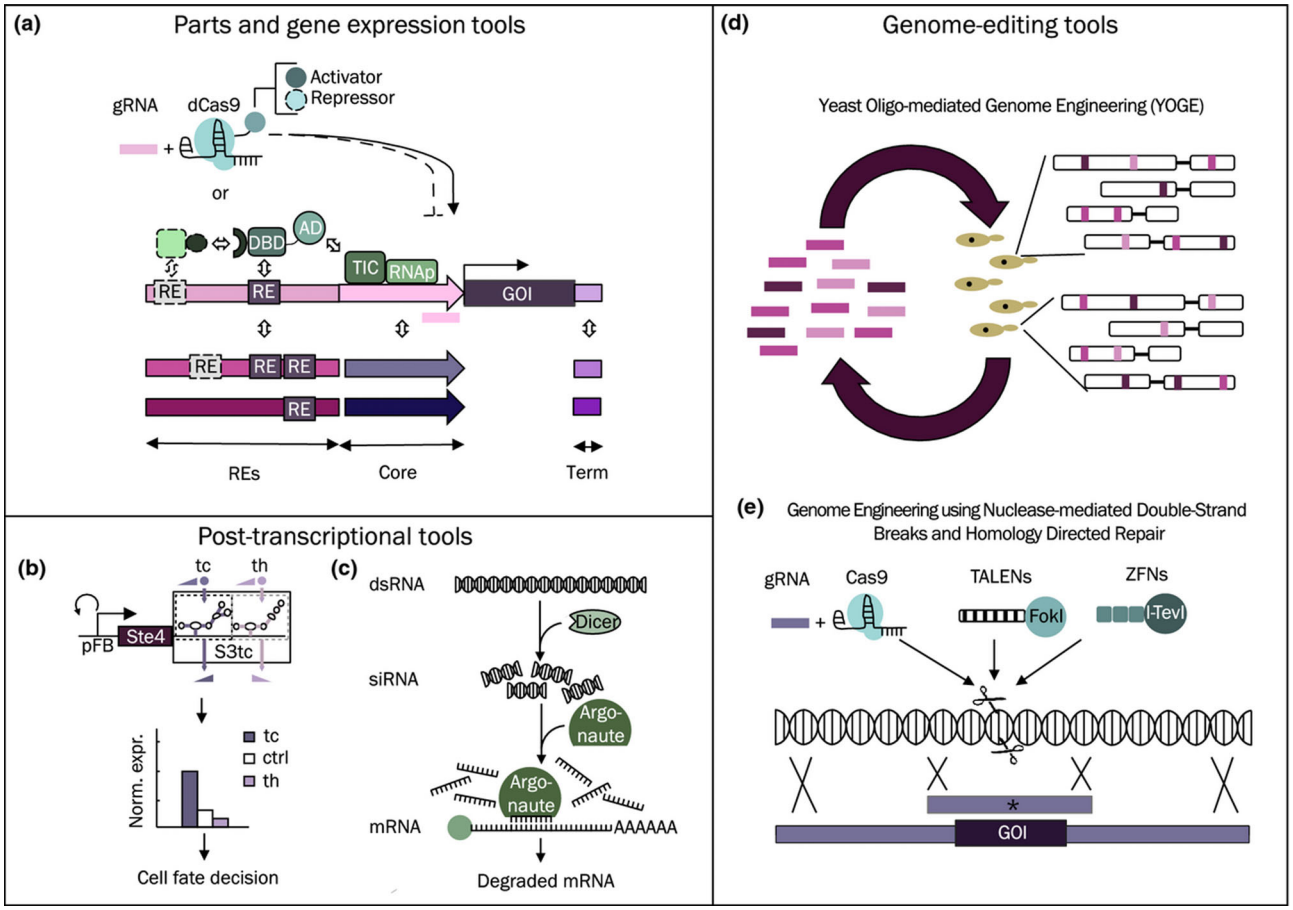

Figure 1. Schematic outline of current synthetic biology tools recently applied in yeast. (a) Modular assembly of synthetic eukaryotic transcription functions. Fusion of catalytically inactive Cas9 nuclease (dCas9) to transcriptional regulatory domains can either increase or decrease transcript abundance of genes targeted by cognate guide RNAs (gRNA) (Gilbert *et al.*, 2013). Likewise, eukaryotic TFs are modular entities that make sequence-specific contacts with DNA and activate transcription through their DNA-binding domain (DBD) and activation domain (AD), respectively. The use of orthogonal DBDs binding to defined REs permits a fully decomposed and tunable design of synthetic TFs which can be wired with other TFs to tune cooperativity and thereby specificity of targeted transcriptional regulation. Also, the strength of the AD can be used to tune transcriptional output from a gene of interest (GOI) together with the transcription initiation complex (TIC) and RNA polymerase II (RNAp) (Khalil *et al.*, 2012). The independent control of these molecular properties enables systematic construction and modulation of transcriptional behavior. White double-headed arrows indicate all the modular and tunable parts that can be exchanged. (b) Schematic of the composite RNA transducer S3tc designed using riboswitches to reduce Ste4 expression and thereby control cell fate in response to theophylline (th) and increase expression in response to tetracycline (tc) (Galloway *et al.*, 2013). (c) Key components of a synthetic RNAi pathway in yeast where double-stranded RNA (dsRNA) with homology to a target gene is degraded by Dicer and the resulting small interfering RNAs (siRNA) are then used by Argonaute to recognize, cleave, and degrade the messenger RNA (mRNA) of the target gene (Crook *et al.*, 2013). (d) YOGE uses short oligos to modify yeast genomes at targeted locations. Iterative rounds can be used to tune insertion frequency and diversity of the genomic library (DiCarlo *et al.*, 2013a,b). (e) Overview of three nuclease-mediated genome editing tools. Cas9 has inherent nuclease activity and uses a short guide RNA (gRNA) to direct a double-strand break (DiCarlo *et al.*, 2013a,b). Transcription activator-like effector nucleases (TALENs) are composed of individual TALE repeats containing 33-35 amino acids that recognize a single base pair via two hypervariable residues. When fused to the FokI nuclease domain, TALENs will target sites consisting of two TALE binding sites separated by a 5- to 7-bp spacer sequence recognized by the FokI cleavage domain spacer sequence (Li *et al.*, 2011). A zinc-finger nuclease (ZFN) composed of customizable zinc-finger repeats fused with the nuclease domain I-TevI will target sites consisting of two zinc-finger binding sites separated by a spacer sequence recognized by the I-TevI cleavage (Kleinstiver *et al.*, 2012). Nuclease-induced DNA double-strand breaks (DSBs) can be repaired by homology-directed repair in the presence of a variant donor fragment with extended homology arms.

Taken together, these examples demonstrate the potential for native and synthetic parts to fine-tune transcriptional regulation for metabolic flux balancing.

Transcription factors (TFs)

Inherent to the characterization of REs of promoters is the need to understand the transcriptional regulators controlling promoter activity. TFs are modular entities performing complex and combinatorial transcriptional regulation. Besides DNA-binding domains (DBDs) and transcriptional activation domains (ADs), TFs can also have sensory (i.e. redox, metabolite binding) domains (Craven *et al.*, 2009; Purcell *et al.*, 2014). The modular nature of TFs makes them ideally suited to orthogonally regulate synthetic transcriptional circuits in yeast. In a hallmark study, Khalil *et al.* (2012) rationally engineered zinc-finger TFs with different DNA specificities and protein-protein interaction propensities, which allowed them to tune promoter strength and the cooperative response (Fig. 1a). In a similar manner, transcription can be regulated using the CRISPR/Cas system of *Streptococcus pyogenes* (Bhaya *et al.*, 2011; Wiedenheft *et al.*, 2012). By simple engineering of guide RNAs (gRNAs) with base-pairing complementarity to target DNA sites, a catalytically dead Cas9 (dCas9)-lacking endonuclease activity can be targeted to different positions in natural yeast promoters and thereby repress their

Table 1. Applied synthetic biology. Selected recent studies applying various synthetic biology tools for improving production titers in *Saccharomyces cerevisiae*.

Product (titer)	Approach	References
Artemisinic acid (25 g L^{-1})	MVA pathway redesign to increase FPP Heterologous expression: *Artemisia annua* amorphadiene synthase and cytochrome P450 Promoter replacement Compartmentalization (FDP synthase and amorphadiene synthase to mitochondria)	Ro *et al.* (2006) *Westfall et al.* (2012) *Farhi et al.* (2011) *Paddon et al.* (2013)
Isobutanol (486 mg L^{-1})	Compartmentalization	Avalos *et al.* (2013)
Miltiradiene (365–488 mg L^{-1})	Protein fusions: SmCPS:SmKSL from *Salvia miltiorrhiza* and BTS1:ERG20	Zhou *et al.* (2012) and Dai *et al.* (2012)
Patchoulol (41 mg L^{-1})	Protein fusion: native farnesyl diphosphate synthase and *Pogostermon cablin* patchoulol synthase	Albertsen *et al.* (2011)
α-santalene (92 mg L^{-1})	Promoter swapping	Scalcinati *et al.* (2012)
Violacein (141 mg L^{-1})	Combinatorial expression library Regression modeling for expression optimization	Lee *et al.* (2013)
β-ionone (0.22 mg L^{-1})	Polycistronic expression cassette Heterologous expression: raspberry dioxygenase (RiCCD1)	Beekwilder *et al.* (2014)
β-amyrin (37.3 mg L^{-1})	Heterologous expression: *Bupleurum falcatum* cytochrome P450 monooxygenase CYP716Y1, oxidosqualene cyclase, and glycosyltransferases Protein fusions: cytochrome P450s CYP716Y1:CYP716A12 and AtATR1:UGT73C11	Moses *et al.* (2014)
Pheromone (195 mg L^{-1})	Protein fusion: *Agrotis segetum* AseD11:AseFAR	Hagström *et al.* (2013)
Oleochemicals (25.3 g L^{-1})	Combinatorial multiplexing of overexpression and knockouts (57 strains) coupled to phenotypic induction	Blazeck *et al.* (2014a,b)
Itaconic acid (>70 mg L^{-1})	RNA interference	Crook *et al.* (2014)
Gurmarin (5 mg L^{-1})	Post-translational modification	Sigoillot *et al.* (2012)
NADH	Protein fusion: cohesin-dockerin pairs	Liu *et al.* (2013)

activity (Fig. 1a) (Farzadfard *et al.*, 2013; Gilbert *et al.*, 2013). Complementary to this, fusion of the SV40 nuclear localization sequence (NLS) and four tandem copies of Herpes Simplex Viral Protein 16 (VP64, a commonly used eukaryotic transcription activator domain) to dCas9 can allow for transcriptional activation of gRNA-targeted reporter promoters (Farzadfard *et al.*, 2013). Extending from these findings, standardized concepts for design of CRISPR-based and other synthetic eukaryotic TFs have been suggested (Purcell *et al.*, 2014). This should accelerate bottom-up approaches to understand design principles of eukaryotic transcriptional regulators and enable the construction of orthogonal and scalable synthetic gene circuits to control gene expression.

Transcription-based sensor-reporter devices

The vast number of methods for saturated mutagenesis and the efficient DNA assembly procedures used for combinatorial library constructions necessitates the development of improved screening and selection tools, particularly when optimizing metabolic pathway flux without easy detectable phenotypes. Biosensors and synthetic sensor–reporter systems hold great promise to increase the throughput of screening assays (Van Dien, 2013).

Again, taking advantage of their modular architecture, researchers have heterologously expressed and engineered synthetic TFs as sensors for detection of defined metabolite levels. Their relatively large dynamic ranges and tuneable input–output functions provide rapid, noninvasive, switchable control of the expression of key enzymatic control points (Hughes *et al.*, 2012). Apart from static control of flux imbalances using combinatorial expression libraries, well-characterized parts have allowed the construction of TF-based dynamic–sensor–reporter systems. Though not as expansively reported in yeast as for bacteria, dynamic transcription-based control devices act by sensing key intermediates to regulate gene expression levels, enabling cells to respond in real time to metabolic flux in accordance with environmental changes (Chou and Keasling, 2013; Dahl *et al.*, 2013). This facilitates efficient consumption of cellular resources, with optimal protein expression levels and chemical production (Stephanopoulos, 2012). In yeast, Teo and Chang applied a non-native TF and synthetic promoters as logic AND gates, enabling transcriptional activation following application of an external signal (e.g. phosphate starvation) and the presence of fatty acid (Teo and Chang, 2014). Such constructs combine inducible pathway expression and pathway balancing by detection of key intermediates.

In another classical synthetic sensor system, *Arabidopsis thaliana* signal synthesis and receptor components were integrated with yeast protein phosphorylation elements and signal responsive promoters into a network under positive-feedback regulation, resulting in population density-dependent gene expression (i.e. quorum sensing) (Chen and Weiss, 2005). While not directly reported for improving chemical production in yeast, sensor–reporter systems and the mathematical models derived from their input–output characteristics will benefit many, different applications of yeast.

In summary, the most recent endeavors of synthetic biology have focused largely on development of transcription regulatory parts. With an increasing reservoir of synthetic sensors and actuators, such parts libraries will enable a much wider range of input-output functions.

Post-transcriptional tools

Although *cis*-encoded transcriptional regulatory DNA parts like promoters are the best characterized regulatory parts, RNA-based co-transcriptional and post-transcriptional aptamers that modulate mRNA secondary structures, stability, and translation efficiency have been applied in yeast. In particular, the Smolke laboratory has developed, characterized, and applied riboswitches to control predefined outputs (Beisel *et al.*, 2011; Michener *et al.*, 2012). These riboswitches include a sensor that is usually comprised of an aptamer specifically interacting with a ligand, and an actuator containing an intrinsic terminator or a ribosome binding site for transcriptional or translational regulation, respectively (Galloway *et al.*, 2013; Wachsmuth *et al.*, 2013; Goler *et al.*, 2014). Taking advantage of the relatively high design modularity and input/output programmability (Win *et al.*, 2009; Liang *et al.*, 2011), Galloway *et al.* (2013) have identified and applied design principles to dynamically control routing of yeast cells to different cell fates. This included the adoption of various tetracyclin- and theophylline-responsive riboswitches (i.e. S3tc) to enhance or reduce *STE4* expression levels, respectively, and thereby control cell fate (Fig. 1b) (Galloway *et al.*, 2013). Although the RNA-based riboswitches so far have been mostly applied for engineering cellular functions (Chen *et al.*, 2010; Culler *et al.*, 2010), synthetic RNA-based riboswitches like these hold the potential to link environmental signals to exogenous control systems. Yet, largely due to the complexity of constructing robust riboswitches with high ligand specificity, applications of riboswitches for improving chemical production in yeast have so far not been reported. However, the capacity of riboswitches to specifically recognize cellular metabolites (i.e. theophylline) has made them attractive components of sensor–actuator systems and hence applicable for the optimization of a broad range of enzymes and metabolic pathways (Michener and Smolke, 2012; Michener *et al.*, 2012). In the future, this should facilitate the translation of these tools to biotechnological applications.

Another post-transcriptional regulatory mechanism recently applied to yeasts is RNA interference (RNAi). By heterologous expression of just the requisite machinery Argonaute and Dicer from the budding yeast *S. castelli* (Drinnenberg *et al.*, 2009), Crook *et al.* (2014) have elucidated optimal designs of hairpin RNA expression cassettes, enabling up to 93% downregulation of a reporter gene's expression level (Fig. 1c) (Crook *et al.*, 2013). As a proof-of-concept for metabolic engineering, the authors elaborated on their earlier observation that *ade3* deletions in yeast can improve the heterologous production of itaconic acid (IA) from *cis*-aconitate during *cis*-aconitate decarboxylase (CAD1) overexpression (Blazeck *et al.*, 2014a,b). Accordingly, Crook *et al.* (2014) targeted *ADE3* knockdown of several *S. cerevisiae* strains and identified commercial Sigma 10560-4A to be the best strain for IA production (Table 1). A major benefit of this system is the portable nature, enabling multiplex downregulation of genes in both standard and unsequenced industrial yeast strains.

Finally, Beekwilder *et al.* (2014) have presented another recent example of a tool for post-transcriptional processing of multigene expression casettes. Although yeast does not offer the same ease of multigene expression cassettes natively present in prokaryotes, Beekwilder *et al.* (2014) were able to achieve polycistronic expression of several enzymes from a single transcript (Beekwilder *et al.*, 2014). Between the individual proteins, the researchers inserted T2A sequences of the *Thosea asigna* virus, enabling efficient production of the beta-carotene biosynthetic enzymes from the carotenoid-producing ascomycete *Xanthophyllomyces dendrorhous* (Beekwilder *et al.*, 2014). Similarly, Unkles *et al.* (2014) generated a large, polycistronic mRNA for the entire penicillin biosynthesis pathway using viral 2A peptide sequences to direct successful co-translational cleavage of pathway enzymes (Unkles *et al.*, 2014).

METABOLIC PATHWAY TOOLS: COMPARTMENTALIZATION, PROTEIN SCAFFOLDS, TAGS, AND FUSIONS

Another paradigm for controlling fluxes and metabolic pathways is spatio-temporal separation of molecular components (Good *et al.*, 2011; Zalatan *et al.*, 2012). This is particularly relevant for gene circuits based on heterologous expression of multienzymatic cascades. Here, spatial engineering efforts seek to force components of a specific multistep enzymatic pathway into physical proximity with the intention of preventing loss of intermediates to diffusion or competing pathways and decreasing the transit times of intermediates (Miles *et al.*, 1999).

Spatial engineering using protein scaffolds is relatively simple to design as it relies on the fusion of known protein-interaction domains to recruit target proteins (Lee *et al.*, 2012). Using protein scaffolds, Dueber *et al.* (2009) optimized the stoichiometry of three mevalonate biosynthetic enzymes recruited to a synthetic scaffold in *Escherichia coli*, thereby achieving a 77-fold improvement in mevalonate titer (Dueber *et al.*, 2009). Using yeast as a host, Liu *et al.* (2013) recently reported the co-immobilization of three dehydrogenases on a trifunctional protein scaffold displayed on the yeast surface facilitating substrate channeling that resulted in a fivefold higher NADH production rate compared to the nonscaffolded enzymes (Liu *et al.*, 2013). In addition to scaffolds, several dimeric fusion proteins have been constructed for increasing substrate channeling. In *S. cerevisae*, Zhou *et al.* (2012) reached miltiradiene titers of 365 mg L^{-1} by improving supply of GGPP through overexpression of a fusion gene of FPP synthase (*ERG20*) and endogenous GGPP synthase (*BTS1*) (Zhou *et al.*, 2012), whereas Dai *et al.* (2012), reached miltiradiene titers of 488 mg L^{-1} by fed-batch fermentation of a strain carrying the fusion of ERG20 with GGPP synthase. Also, Albertsen *et al.* (2011) showed that a hybrid protein consisting of *S. cerevisiae* native FPPS and *Pogosternon cablin* patchoulol synthase (PTS) increased the production of the sesquiterpene patchoulol up to twofold compared to nonfused versions of FPPS and PTS (Albertsen *et al.*, 2011). Likewise, using a 2A oligopeptide (De Felipe *et al.*, 2006), the cytochrome P450s CYP716Y1 and CYP716A12 were linked to produce a self-processing polyprotein for oxidation of β-amyrin (Moses *et al.*, 2014). In addition to fusions, tagging sweet-response inhibitor gurmarin in *Pichia pastoris* with the α-factor preprosequence from *S. cerevisae* was shown to improve gurmarin production titers (Sigoillot *et al.*, 2012), whereas genes involved in post-translational modifications like ubiquitin-mediated protein degradation were shown to be indispensible for butanol tolerance, a prerequisite for improved product titers in *S. cerevisae* (González-Ramos *et al.*, 2013).

Another strategy to achieve greater local enzyme and intermediate concentrations is by compartmentalization of metabolic pathways into the mitochondrion. As with protein scaffolds, higher concentrations of enzymes, substrates, and cofactors within the targeted compartment could improve titer, rate, and yield figures. Indeed, Avalos *et al.* (2013) recently improved fusel alcohol (isobutanol, isopentanol, and 2-methyl-1-butanol) production in *S. cerevisae* by 260% by compartmentalizing the valine Ehrlich degradation pathway, attaching the

N-terminal mitochondrial localization signal (MLS) from subunit IV of the yeast cytochrome c oxidase (CoxIV) to the target enzymes (Avalos *et al.*, 2013). Similarly, tagging *Citrus sinensis* valencene synthase (*CsTPS1*) with the MLS of mitochondrial *COX4*, Farhi *et al.* (2011) harnessed the mitochondrial FPP pool to increase valencene titers threefold compared to that generated via the use of cytosolic *CsTPS1* (Farhi *et al.*, 2011). Overall, these studies suggest that the spatial organization of metabolic enzymes in the yeast mitochondrion can greatly improve chemical production, although other studies report no improvement from similar approaches to boost IA production (Blazeck *et al.*, 2014a,b).

Another spatial engineering effort has been reported by Yang *et al.* (2013). Here, the authors used an optogenetic design, based on a light-gated protein dimerization system, to achieve temporal, rapid, reversible, and titratable localization of a candidate protein into eight different organelles (Yang *et al.*, 2013). In this way, they were able to control the spatial and temporal dynamics of Gal80 nuclear depletion and thereby galactose signaling activation. Such light-inducible systems represent a powerful approach for spatio-temporal control of key metabolic pathways, and furthermore for achieving a better understanding of complex biological systems (Yang *et al.*, 2013).

In summary, together with gene expression-based pathway optimizations like targeted knockouts, knockdowns, and overexpression, recent advances in protein targeting, scaffolding, and compartmentalization of enzymes have been shown to improve production of desired molecules.

CHROMOSOME EDITING TOOLS

Generation of DNA parts libraries by error-prone PCR or targeted mutagenesis followed by directed evolution approaches, in which a non-natural selective pressure is applied to the parts library to identify a desired trait, have been used to evolve synthetic devices and systems and expand the genomic diversity (Tyo *et al.*, 2011; Rajkumar *et al.*, 2013; Ellefson *et al.*, 2014). In yeast, this has been applied to re-engineer the tryptophanyl tRNA-synthetase:suppressor tRNA pair from *S. cerevisiae* to efficiently and site specifically incorporate an unnatural amino acid into proteins (Ellefson *et al.*, 2014) and to evolve a T7 RNA polymerase variant that recognizes an orthogonal promoter (Ellefson *et al.*, 2014). Given a feasible and robust screening system, large libraries can be screened and more orthogonal and artificial enzymes with new functions (i.e. substrate specificities) can be selected in high-throughput manners (Trudeau *et al.*, 2013).

In addition to such gene- and promoter-centric approaches, multiplex automated genome engineering (MAGE) allows targeted, multiplexed editing, and evolution of microbial genomes using short oligonucleotides that are used as templates for allelic replacements mediated by the bacteriophage λ-Red ssDNA-binding protein β (Wang *et al.*, 2009). In *E. coli,* this technique has been applied to optimize the DXP pathway to overproduce the industrially important isoprenoid lycopene and improve biosynthesis of aromatic amino acid derivatives (Wang *et al.*, 2009, 2012). In an analogous manner, multiplexed yeast oligo-mediated genome engineering (YOGE) offers the possibility to simultaneously edit multiple genomic loci using variant donor templates and harness the endogenous recombination machinery of yeast (Fig. 1d) (DiCarlo *et al.*, 2013a,b). By multiplexing and the cyclical nature of YOGE/MAGE, this approach offers the possibility to rapidly generate a diverse set of genetic changes and to control the frequency of insertions by the numbers of cycles applied. In yeast, DiCarlo *et al.*, 2013a,b optimized key parameters for YOGE and achieved 0.2–2.0% oligonucleotide insertion efficiencies without selection for the modification, making it suitable for moderate screening efforts with an estimated library size of 10^2–10^5 recombinants per locus per cycle (Fig. 1d) (DiCarlo *et al.*, 2013a,b). Most importantly, MAGE and YOGE allow for enrichment of desired genotypes with the ability to rapidly cycle the procedure and thereby obtain higher frequencies.

Just prior to publishing the YOGE data, DiCarlo *et al.*, 2013a,b applied the CRISPR–Cas9 system to make targeted knockout of single genes, including the negative selectable marker *CAN1*. When co-transforming a guide RNA to target the *CAN1* gene together with a donor oligonucleotide containing 2 bp changes and a premature TAG stop codon in the *CAN1* open reading frame, the authors observed almost 100% recombination frequency without the use of a selectable marker (Fig. 1e). For comparison, without the co-transformation of a donor oligonucleotide, *CAN1* mutants arose with a frequency of 0.07%. Acknowledging the ease of designing site specificity for CRISPR–Cas9 targets via the expression of short gRNAs obviates the need to engineer orthogonal DBDs and/or insert binding sites into predefined genomic loci. Such advantages may make unnecessary other endonuclease-based approaches using TAL, ZF, and homing endonucleases previously applied to yeast (Fig. 1e) (Li *et al.*, 2011; Kleinstiver *et al.*, 2012; Kuijpers *et al.*, 2013).

As DNA synthesis costs decrease and design tools improve, one can imagine a day when the synthetic biologist designs an entirely new chromosome rather than randomly or site specifically engineering small parts of native chromosome. That day is getting closer, as demonstrated by Annaluru *et al.* (2014), who swapped all TAG stop codons with TAA; deleted subtelomeric regions, introns, transfer RNAs, transposons, and silent mating loci; and inserted loxPsym sites to facilitate scrambling of yeast chromosome architecture (Annaluru *et al.*, 2014). Such endeavors suggest that it will soon become feasible to engineer new eukaryotic genomes with specific design constraints, including those optimized for bio-based chemical production.

Taken together, targeted libraries of DNA part variants and oligo-mediated recombineering of microbial genomes continuously expand sequence diversity and are hallmarks for both metabolic engineering and synthetic biology efforts leveraging sequence to function analyses at an ever-increasing speed.

COMPUTATION AND DATA MANAGEMENT: CAD AND REPOSITORIES

One of the main difficulties of genetically altering microorganisms for the production of native and non-native high-value compounds is the identification of the appropriate metabolic pathways capable of transforming raw materials into the targeted compound. With the advent of whole-genome sequencing, and the metabolic networks and genome-scale models derived from the genome sequences (Österlund *et al.*, 2013; Chumnanpuen *et al.*, 2014), computer-aided design (CAD) tools have been developed to generate heterologous pathways predicted to produce the compound of interest *in silico* (for a recent review on CAD tools, readers are referred to (Fernández-Castané *et al.*, 2014). Such tools are important to build novel biosynthetic pathways and to improve flux through existing pathways, and were recently adopted by Misra *et al.* (2013), to elucidate both intuitive and nonintuitive gene targets for improving production of the artemisinin precursor dihydroartemisinic acid. Also, Otero *et al.* (2013), benefitted from CAD tools for improving succinic acid production.

Even with good CAD tools, it will be difficult to predictably engineer complex biological systems without collections of well-characterized parts, whose descriptions can be used in CAD programmes. To this end, nascent biological parts registries have been developed, including the Registry of Biological Parts (http://parts.igem.org/Main_Page) and the Inventory of Composable Elements (JBEI-ICE) (Ham *et al.*, 2012). Unfortunately, parts are often poorly characterized (Kwok, 2010), making it difficult to rationally design even the simplest genetic circuit. To improve usability, Wang *et al.* (2013) recently provided a lookup table to demonstrate the organization of parts used in engineered systems (Wang *et al.*, 2013). As some parts (e.g. RNA aptamers, fluorescent reporters) are orthogonal and can be applied universally, that is host-unspecific, such tables facilitate bottom-up design approaches in synthetic biology. Ultimately, the emphasis on both parts and systems conditionality, and the access to experimentally validated data should maximize our future understanding of the principles of genetic circuit design and minimize time-demanding trial and error experiments metabolic engineers often face.

CONCLUSIONS AND PERSPECTIVES

In this review, we have described a current status of the most recent parts and device usability, emphasized genome editing strategies, presented the current spatial engineering efforts and data needs for improved CAD performance. Most of the tools described have already helped improve chemical and fuels production in yeast, and others hold great promises for bridging the discovery to application gap in the nearest future.

However, despite major progress in applying synthetic biology for chemical and fuels production, significant challenges still hamper the predictability of the design-build-test cycle. These hurdles mainly include poor predictive models due to inherent nonlinearity of biological systems, a lack of widely applicable and scalable parts libraries, and low-throughput characterization techniques. As such, it is evident that synthetic biology is still in its infancy, particularly when it comes to applications.

To improve predictability of build performance for industry-relevant bio-based production, better algorithms are needed. Inherent is the need for the scientific community to allocate more resources for gathering of omics training data, sampled from relevant growth and production conditions (most relevant carbon source, fermentation strategies, environmental conditions, etc.). For this to be of true mutual benefit to both academia (understanding) and industry (applicability), open communication about general bioprocess bottlenecks, basic feedstock, and fermentation conditions needs to be communicated. Additionally, to account for extrinsic noise in part and device performances across laboratories, incorporation of a normalizing construct to improve consistency of data related to such performances (Kelly *et al.*, 2009)—ultimately providing comprehensive, high-quality, quantitative data sets deposited into open-access registries—is instrumental for improved CAD performance. This also includes the further development of systematic parts collections with minimum information about parts behavior.

With such emphasis on unbiased learning from sequence to function metrics, and multiparametric interrogation of industry-relevant phenotype to genotype relations, synthetic biology should make the design-test-build workflow more tangible (Fig. 2). Here, researchers and engineers will use CAD software platforms to design and test a virtual organism, and *then* build its genome. Although several companies and some

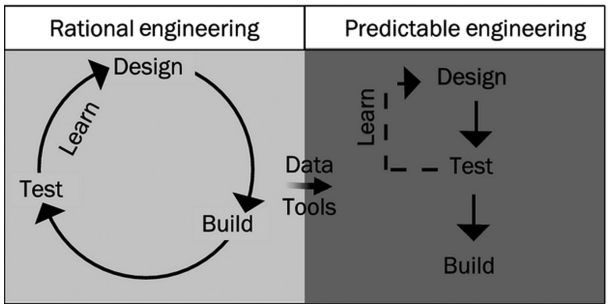

Figure 2. Using multidimensional data and high-throughput screens, synthetic biology seeks a transformation from the current rational design-build-test cycle to a predictable design-test-build workflow.

academic institutions are applying a design-build-test cycle (Van Dien, 2013), the design-test-build workflow is instinctively more cost-effective and rational than retrofitting designs following labor-intensive build procedures (Fig. 2) (Ellis *et al.*, 2009; Lee *et al.*, 2013). Again, investing resources in parts prospecting, high-speed DNA synthesis, and data feeding from systems biology should improve our basic understanding of biological systems and chemical interactions, and drastically help synthetic biology to speed metabolic engineering efforts for future yeast-based cell factories.

ACKNOWLEDGEMENTS

The authors wish to thank the Novo Nordisk Foundation for financial support. The authors declare no conflict of interests.

Conflict of interest statement. None declared.

REFERENCES

Albertsen L, Chen Y, Bach LS, Rattleff S, Maury J, Brix S, Nielsen J, Mortensen UH Diversion of flux toward sesquiterpene production in *Saccharomyces cerevisiae* by fusion of host and heterologous enzymes. *Appl Environ Microbiol* 2011;**77**:1033–40.

Annaluru N, Muller H, Mitchell LA, *et al.* Total synthesis of a functional designer eukaryotic chromosome. *Science (80-.)* 2014;**344**:55–8.

Avalos JL, Fink GR, Stephanopoulos G Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nat Biotechnol* 2013;**31**:335–41.

Beekwilder J, van Rossum MH, Koopman F, *et al.* Polycistronic expression of a β-carotene biosynthetic pathway in *Saccharomyces cerevisiae* coupled to β-ionone production. *J Biotechnol* 2014 http://www.ncbi.nlm.nih.gov/pubmed/24486029 (Accessed February 25, 2014).

Beisel CL, Chen YY, Culler SJ, Hoff KG, Smolke CD Design of small molecule-responsive microRNAs based on structural requirements for Drosha processing. *Nucleic Acids Res* 2011;**39**:2981–94.

Bhaya D, Davison M, Barrangou R CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet* 2011;**45**:273–97.

Blazeck J, Garg R, Reed B, Alper HS Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters. *Biotechnol Bioeng* 2012;**109**:2884–95.

Blazeck J, Reed B, Garg R, Gerstner R, Pan A, Agarwala V, Alper HS Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 2013;**97**:3037–52.

Blazeck J, Hill A, Liu L, Knight R, Miller J, Pan A, Otoupal P, Alper HS Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat Commun* 2014a;**5**:3131.

Blazeck J, Miller J, Pan A, Gengler J, Holden C, Jamoussi M, Alper HS Metabolic engineering of *Saccharomyces cerevisiae* for itaconic acid production. *Appl Microbiol Biotechnol* 2014b http://www.ncbi.nlm.nih.gov/pubmed/24997118 (Accessed July 6, 2014).

Borodina I, Nielsen J Advances in metabolic engineering of yeast *Saccharomyces cerevisiae* for production of chemicals. *Biotechnol J* 2014 http://doi.wiley.com/10.1002/biot.201300445 (Accessed March 22, 2014).

Buchholz K, Collins J The roots-a short history of industrial microbiology and biotechnology. *Appl Microbiol Biotechnol* 2013;**97**:3747–62.

Chen M-T, Weiss R Artificial cell-cell communication in yeast *Saccharomyces cerevisiae* using signaling elements from *Arabidopsis thaliana*. *Nat Biotechnol* 2005;**23**:1551–5.

Chen YY, Jensen MC, Smolke CD Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *P Natl Acad Sci USA* 2010;**107**:8531–6.

Chou HH, Keasling JD Programming adaptive control to evolve increased metabolite production. *Nat Commun* 2013;**4**:2595.

Chumnanpuen P, Hansen MAE, Smedsgaard J, Nielsen J Dynamic metabolic footprinting reveals the key components of metabolic network in yeast *Saccharomyces cerevisiae*. *Int J Genomics* 2014;**2014**:894296.

Craven SH, Ezezika OC, Haddad S, Hall RA, Momany C, Neidle EL Inducer responses of BenM, a LysR-type transcriptional regulator from *Acinetobacter baylyi* ADP1. *Mol Microbiol* 2009;**72**:881–94.

Crook NC, Schmitz AC, Alper HS Optimization of a yeast RNA interference system for controlling gene expression and enabling rapid metabolic engineering. *ACS Synth Biol* 2013 http://www.ncbi.nlm.nih.gov/pubmed/24328131 (Accessed February 24, 2014).

Crook NC, Schmitz AC, Alper HS Optimization of a yeast RNA interference system for controlling gene expression and enabling rapid metabolic engineering. *ACS Synth Biol* 2014;**3**:307–13.

Culler SJ, Hoff KG, Smolke CD Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. *Science* 2010;**330**:1251–5.

Curran KA, Karim AS, Gupta A, Alper HS Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications. *Metab Eng* 2013;**19**:88–97.

Dahl RH, Zhang F, Alonso-Gutierrez J, *et al.* Engineering dynamic pathway regulation using stress-response promoters. *Nat Biotechnol* 2013;**31**:1039–46.

Dai Z, Liu Y, Huang L, Zhang X Production of miltiradiene by metabolically engineered *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2012;**109**:2845–53.

De Felipe P, Luke GA, Hughes LE, Gani D, Halpin C, Ryan MD E unum pluribus: multiple proteins from a self-processing polyprotein. *Trends Biotechnol* 2006;**24**:68–75.

DiCarlo JE, Conley AJ, Penttilä M, Jäntti J, Wang HH, Church GM Yeast oligo-mediated genome engineering (YOGE). *ACS Synth Biol* 2013a http://www.ncbi.nlm.nih.gov/pubmed/24160921 (Accessed February 26, 2014).

DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res* 2013b;**41**:4336–43.

Drinnenberg IA, Weinberg DE, Xie KT, Mower JP, Wolfe KH, Fink GR, Bartel DP RNAi in budding yeast. *Science* 2009;**326**:544–50.

Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KLJ, Keasling JD Synthetic protein scaffolds provide modular control over metabolic flux. *Nat Biotechnol* 2009;**27**:753–9.

Ellefson JW, Meyer AJ, Hughes R a, Cannon JR, Brodbelt JS, Ellington AD Directed evolution of genetic parts and circuits by compartmentalized partnered replication. *Nat Biotechnol* 2014;**32**:97–101.

Ellis T, Wang X, Collins JJ Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nat Biotechnol* 2009;**27**:465–71.

Farhi M, Marhevka E, Masci T, Marcos E, Eyal Y, Ovadis M, Abeliovich H, Vainstein A Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab Eng* 2011;**13**:474–81.

Farzadfard F, Perli SD, Lu TK Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth Biol* 2013;**2**:604–13.

Fernández-Castané A, Fehér T, Carbonell P, Pauthenier C, Faulon J-L Computer-aided design for metabolic engineering. *J Biotechnol* 2014 http://www.ncbi.nlm.nih.gov/pubmed/24704607 (Accessed April 3, 2014).

Galloway KE, Franco E, Smolke CD Dynamically reshaping signaling networks to program cell fate via genetic controllers. *Science* 2013;**341**:1235005.

Gilbert LA, Larson MH, Morsut L, *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;**154**:442–51.

Goler JA, Carothers JM, Keasling JD Dual-selection for evolution of *in vivo* functional aptazymes as riboswitch parts. *Methods Mol Biol* 2014;**1111**:221–35.

González-Ramos D, van den Broek M, van Maris AJ, Pronk JT, Daran J-MG Genome-scale analyses of butanol tolerance in *Saccharomyces cerevisiae* reveal an essential role of protein degradation. *Biotechnol Biofuels* 2013;**6**:48.

Good MC, Zalatan JG, Lim WA Scaffold proteins: hubs for controlling the flow of cellular information. *Science* 2011;**332**:680–86.

Hagström Å, Wang HL, Liénard MA, Lassance JM, Johansson T, Löfstedt C A moth pheromone brewery: production of (Z)-11-hexadecenol by heterologous co-expression of two biosynthetic genes from a noctuid moth in a yeast cell factory. *Microb Cell Fact* 2013;**12**:125.

Ham TS, Dmytriv Z, Plahar H, Chen J, Hillson NJ, Keasling JD Design, implementation and practice of JBEI-ICE: an open source biological part registry platform and tools. *Nucleic Acids Res* 2012;**40**:e141.

Hong K-K, Nielsen J Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. *Cell Mol Life Sci* 2012;**69**:2671–90.

Hubmann G, Thevelein JM, Nevoigt E Natural and modified promoters for tailored metabolic engineering of the yeast *Saccharomyces cerevisiae*. *Methods Mol Biol* 2014;**1152**:17–42.

Hughes RM, Bolger S, Tapadia H, Tucker CL Light-mediated control of DNA transcription in yeast. *Methods* 2012;**58**:385–91.

Ito Y, Yamanishi M, Ikeuchi A, Imamura C, Tokuhiro K, Kitagawa T, Matsuyama T Characterization of five terminator regions that increase the protein yield of a transgene in *Saccharomyces cerevisiae*. *J Biotechnol* 2013;**168**:486–92.

Kelly JR, Rubin AJ, Davis JH, Ajo-Franklin CM, Cumbers J, Czar MJ, de Mora K, Glieberman AL, Monie DD, Endy D, *et al*. Measuring the activity of BioBrick promoters using an *in vivo* reference standard. *J Biol Eng* 2009;**3**:4.

Khalil AS, Lu TK, Bashor CJ, Ramirez CL, Pyenson NC, Joung JK, Collins JJ A synthetic biology framework for programming eukaryotic transcription functions. *Cell* 2012;**150**:647–58.

Kleinstiver BP, Wolfs JM, Kolaczyk T, Roberts AK, Hu SX, Edgell DR Monomeric site-specific nucleases for genome editing. *P Natl Acad Sci USA* 2012;**109**:8061–6.

Kotula JW, Kerns SJ, Shaket LA, Siraj L, Collins JJ, Way JC, Silver PA Programmable bacteria detect and record an environmental signal in the mammalian gut. *P Natl Acad Sci USA* 2014;**111**:4838–43.

Kuijpers NGA, Chroumpi S, Vos T, Solis-Escalante D, Bosman L, Pronk JT, Daran J-M, Daran-Lapujade P One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2013;**13**:769–81.

Kwok R Five hard truths for synthetic biology. *Nature* 2010;**463**:288–90.

Lan EI, Liao JC Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. *Bioresour Technol* 2013;**135**:339–49.

Lee H, DeLoache WC, Dueber JE Spatial organization of enzymes for metabolic engineering. *Metab Eng* 2012;**14**:242–51.

Lee ME, Aswani A, Han AS, Tomlin CJ, Dueber JE Expression-level optimization of a multi-enzyme pathway in the absence of a high-throughput assay. *Nucleic Acids Res* 2013;**41**:10668–78

Li T, Huang S, Jiang WZ, Wright D, Spalding MH, Weeks DP, Yang B TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Res* 2011;**39**:359–72.

Liang JC, Bloom RJ, Smolke CD Engineering biological systems with synthetic RNA molecules. *Mol Cell* 2011;**43**:915–26.

Liu F, Banta S, Chen W Functional assembly of a multi-enzyme methanol oxidation cascade on a surface-displayed trifunctional scaffold for enhanced NADH production. *Chem Commun (Camb)* 2013;**49**:3766–8.

Marchisio MA *In silico* design and *in vivo* implementation of yeast gene Boolean gates. *J Biol Eng* 2014;**8**:6.

Michener JK, Smolke CD High-throughput enzyme evolution in *Saccharomyces cerevisiae* using a synthetic RNA switch. *Metab Eng* 2012;**14**:306–16.

Michener JK, Thodey K, Liang JC, Smolke CD Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab Eng* 2012;**14**:212–22.

Miles EW, Rhee S, Davies DR The molecular basis of substrate channeling. *J Biol Chem* 1999;**274**:12193–6.

Misra A, Conway MF, Johnnie J, Qureshi TM, Lige B, Derrick AM, Agbo EC, Sriram G Metabolic analyses elucidate non-trivial gene targets for amplifying dihydroartemisinic acid production in yeast. *Front Microbiol* 2013;**4**:200.

Moser F, Broers NJ, Hartmans S, Tamsir A, Kerkman R, Roubos JA, Bovenberg R, Voigt CA Genetic circuit performance under conditions relevant for industrial bioreactors. *ACS Synth Biol* 2012;**1**:555–64.

Moses T, Pollier J, Almagro L, Buyst D, Van Montagu M, Pedreño MA, Martins JC, Thevelein JM, Goossens A Combinatorial biosynthesis of sapogenins and saponins in *Saccharomyces cerevisiae* using a C-16α hydroxylase from *Bupleurum falcatum*. *P Natl Acad Sci USA* 2014;**111**:1634–9.

Nielsen AAK, Segall-Shapiro TH, Voigt CA Advances in genetic circuit design: novel biochemistries, deep part mining, and precision gene expression. *Curr Opin Chem Biol* 2013;**17**:878–92.

Österlund T, Nookaew I, Bordel S, Nielsen J Mapping condition-dependent regulation of metabolism in yeast through genome-scale modeling. *BMC Syst Biol* 2013;**7**:36.

Otero JM, Cimini D, Patil KR, Poulsen SG, Olsson L, Nielsen J Industrial systems biology of *Saccharomyces cerevisiae* enables novel succinic acid cell factory. *PLoS One* 2013;**8**:e54144.

Paddon CJ, Keasling JD Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nat Rev Microbiol* 2014;**12**:355–67.

Paddon CJ, Westfall PJ, Pitera DJ, *et al*. High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 2013;**496**:528–32.

Paradise EM, Kirby J, Chan R, Keasling JD Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by down-regulating squalene synthase. *Biotechnol Bioeng* 2008;**100**:371–8.

Partow S, Siewers V, Bjørn S, Nielsen J, Maury J Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast* 2010;**27**:955–64.

Purcell O, Peccoud J, Lu TK *Rule-Based Design of Synthetic Transcription Factors in Eukaryotes* 2014 American Chemical Society http://pubs.acs.org/doi/ipdf/10.1021/sb400134k (Accessed March 19, 2014).

Rajkumar AS, Dénervaud N, Maerkl SJ Mapping the fine structure of a eukaryotic promoter input-output function. *Nat Genet* 2013;**45**:1207–15.

Ro DK, Paradise EM, Ouellet M, *et al*. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 2006;**440**:940–3.

Scalcinati G, Knuf C, Partow S, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. *Metab Eng* 2012;**14**:91–103.

Shin S-Y, Jung S-M, Kim M-D, Han NS, Seo J-H Production of resveratrol from tyrosine in metabolically engineered *Saccharomyces cerevisiae*. *Enzyme Microb Technol* 2012;**51**:211–6.

Siddiqui MS, Thodey K, Trenchard I, Smolke CD Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools. *FEMS Yeast Res* 2012;**12**:144–70.

Sigoillot M, Brockhoff A, Lescop E, Poirier N, Meyerhof W, Briand L Optimization of the production of gurmarin, a sweet-taste-suppressing protein, secreted by the methylotrophic yeast *Pichia pastoris*. *Appl Microbiol Biotechnol* 2012;**96**:1253–63.

Stephanopoulos G Synthetic biology and metabolic engineering. *ACS Synth Biol* 2012;**1**:514–25.

Teo WS, Chang MW Development and characterization of AND-gate dynamic controllers with a modular synthetic GAL1 core promoter in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2014;**111**:144–51.

Trudeau DL, Smith MA, Arnold FH Innovation by homologous recombination. *Curr Opin Chem Biol* 2013;**17**:902–9.

Tyo KEJ, Nevoigt E, Stephanopoulos G Directed evolution of promoters and tandem gene arrays for customizing RNA synthesis rates and regulation. *Methods Enzymol* 2011;**497**:135–55.

Unkles SE, Valiante V, Mattern DJ, Brakhage AA Synthetic biology tools for bioprospecting of natural products in eukaryotes. *Chem Biol* 2014 http://www.ncbi.nlm.nih.gov/pubmed/24631120 (Accessed March 21, 2014).

Van Dien S From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. *Curr Opin Biotechnol* 2013;**24**:1061–8.

Wachsmuth M, Findeiβ S, Weissheimer N, Stadler PF, Mörl M *De novo* design of a synthetic riboswitch that regulates transcription termination. *Nucleic Acids Res* 2013;**41**: 2541–51.

Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM Programming cells by multiplex genome engineering and accelerated evolution. *Nature* 2009;**460**:894–8.

Wang HH, Kim H, Cong L, Jeong J, Bang D, Church GM Genome-scale promoter engineering by coselection MAGE. *Nat Methods* 2012;**9**:591–3.

Wang Y-H, Wei KY, Smolke CD Synthetic biology: advancing the design of diverse genetic systems. *Annu Rev Chem Biomol Eng* 2013;**4**:69–102.

Westfall PJ, Pitera DJ, Lenihan JR, *et al*. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *P Natl Acad Sci USA* 2012;**109**:E111–8.

Wiedenheft B, Sternberg SH, Doudna JA RNA-guided genetic silencing systems in bacteria and archaea. *Nature* 2012;**482**:331–8.

Win MN, Liang JC, Smolke CD Frameworks for programming biological function through RNA parts and devices. *Chem Biol* 2009;**16**:298–310.

Yamanishi M, Ito Y, Kintaka R, Imamura C, Katahira S, Ikeuchi A, Moriya H, Matsuyama T A genome-wide activity assessment of terminator regions in *Saccharomyces cerevisiae* provides a-terminatome-toolbox. *ACS Synth Biol* 2013 http://www.ncbi.nlm.nih.gov/pubmed/23654277 (Accessed March 16, 2014).

Yang X, Jost AP-T, Weiner OD, Tang C A light-inducible organelle-targeting system for dynamically activating and inactivating signaling in budding yeast. *Mol Biol Cell* 2013;**24**:2419–30.

Zalatan JG, Coyle SM, Rajan S, Sidhu SS, Lim WA Conformational control of the Ste5 scaffold protein insulates against MAP kinase misactivation. *Science* 2012;**337**:1218–22.

Zhou YJ, Gao W, Rong Q, *et al*. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. *J Am Chem Soc* 2012;**134**:3234–41.