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Molecular tools for chemical biotechnology

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Reference Annotations [7]*The authors developed a bacterial promoter library with relative protein production rates that are independent of the coding sequence of the expressed protein. This is one of the first examples of a context-independent biological control element.
[11]**Researchers engineered an RNA-based scaffolding system capable of forming 1, 2, or 3-D assemblies *in vivo* and binding tagged enzymes with aptamers.
[16]*By fusing plant sesquiterpene synthases to mitochondrial targeting sequences, the authors co-localized these enzymes with host enzymes that produce sesquiterpene precursors and thereby improved sesquiterpene biosynthesis in engineered yeast.
[20]**In this work, researchers replaced entire chromosome arms in yeast with designed synthetic versions. This is the first example of 10-100,000 bp sections of synthetic DNA being constructed to replace native sequence in a eukaryote.
[29]*The authors identified a biocatalytic route to 1,4-butanediol from central metabolism using pathway prediction software, built the suggested pathways, and improved overall titers by using a whole-cell metabolic model of *E. coli* to predict engineering targets. This is one of the first successful applications of *de novo* pathway prediction software.
[31]*Researchers improved titers of *n*-butanol by carefully identifying and characterizing enzymes from genetic databases with optimal kinetic parameters. This work is an excellent example of how genetic information can be rationally implemented for pathway design.
[33]**Genetic databases were used to identify a 36 kb *Vibrio splendidus* genome fragment suspected to encode transporters needed for alginate utilization, and this large fragment was cloned into *E. coli* by screening a genomic fosmid library. The researchers also engineered an alginate lyase secretion system and heterologous ethanol pathway to produce ethanol from macroalgae feedstocks.
[37]*The authors used conjugative genome assembly in *E. coli* to combine 32 defined sections of different strain genomes into a single recoded *E. coli* genome.
[43]**An *in vivo* RNA-based biosensor was used to couple intracellular small-molecule product concentrations to GFP levels, allowing a large enzyme library to be rapidly screened by plate-based fluorescence readings and FACS. This is one of the first screening methods that does not depend on any innate property of the substrate, product, or enzyme.
[44]**In the system developed here, the expression of a protein necessary for phage survival is linked to a desired biomolecule activity. The host *E. coli* is infected by selection phage that encode the biomolecule library, and the culture is maintained in a turbidostat such that phage that encode biomolecule library members that activate the expression of the phage-survival protein are enriched over time.
[48]**By measuring changes to global transcriptional patterns by DNA microarrays, heme depletion was discovered to be the principal source of stress for a yeast strain expressing evolved P450 monooxygenase enzymes. Subsequently alleviating this stress by overexpression of the genes responsible for heme biosynthesis showed recovered activity for the evolved P450 at high copy expression.
[53]**Model-guided analysis identified gene knockouts for increase in cellular availability of NADPH for sesquiterpene synthesis. The analysis also suggested a gene target to overexpress – this targeted change offset the growth impairment in the engineered strain versus wild type.
[54]**Targeted changes to the native *E. coli* metabolism were identified by a whole cell *E. coli* metabolic model. These modifications increased flux toward lactyl-CoA production, increasing lactate fraction of polymer as well as total polymer content of the cells by dry weight %.
[64]**Expression of a key biosynthetic enzyme in the isoprenoid biosynthesis pathway was tied to media glucose concentration by use of a glucose responsive promoter.
[65]*Heterologous protein expression can be tied to cell concentration by use of modified quorum sensing components.
[67]**The native *E. coli* transcriptional regulator *FadR* was used to control ethanol overproduction and FAEE synthase modules relative to pools of free fatty acids in the *E. coli* host strain. This approach achieved higher titers than tuning relative transcriptional strengths of the different modules with various constitutive promoters.

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Abstract

Biotechnological production of high value chemical products increasingly involves engineering *in vivo* multi-enzyme pathways and host metabolism. Recent approaches to these engineering objectives have made use of molecular tools to advance *de novo* pathway identification, tunable enzyme expression, and rapid pathway construction. Molecular tools also enable optimization of single enzymes and entire genomes through diversity generation and screening, whole cell analytics, and synthetic metabolic control networks. In this review, we focus on advanced molecular tools and their applications to engineered pathways in host organisms, highlighting the degree to which each tool is generalizable.

Introduction

Chemical biotechnology is the use of biocatalysts in engineered systems to produce bulk and fine chemicals [1]. Three waves of biocatalysis have been described: first, realization that biological components could be used for chemical transformations; second, development of genetic engineering techniques needed for industrial production of proteins; and third, development of directed evolution-enabled enzyme engineering [2]. The coming wave relies not only on further improvements in protein engineering and DNA synthesis technologies, but also critically on our ability to engineer controlled, multi-enzyme pathway systems. Although isolated enzymes are widely used industrially today, whole cells are a more feasible system for multi-enzyme pathways. The introduction of heterologous pathways into a host organism and metabolic flux optimization toward the product of interest is a synergistic application of concepts from metabolic engineering and synthetic biology [3]. In this review, we describe selected contributions of metabolic engineering, synthetic biology, systems biology, and protein engineering to chemical biotechnology to improve the productivity of multi-enzyme pathways. These fields have provided advanced molecular tools for *de novo* pathway identification, tuned pathway construction, diversity generation and screening, genome-scale identification of optimization targets, and dynamic pathway control. Here, we focus on such molecular tools developed, improved, and applied in new contexts over the past few years.

Enhanced tools for precise biosynthetic pathway construction

Engineered biosynthetic pathways require composition of genetically encoded expression devices that support precise and tunable levels of pathway enzymes. Both the number of characterized control elements—such as ribosome binding sites (RBSs), promoters, and terminators—and the degree to which those control elements can be made to behave in a predictable manner under a range of contexts have expanded. Additionally, improved and new methods have been developed to assemble these control elements with enzymes to construct biosynthetic pathways.

One challenge to the rational design of genetically encoded elements is that they often behave in a context-dependent manner, exhibiting properties that depend on the combination of other elements used in the device or exhibiting off-target perturbations of the biological host. This challenge has been addressed by designing for context and then iteratively optimizing to improve behavior. Contextual features to consider range from the specific (e.g., DNA sequence surrounding the element) to the holistic (e.g., environmental growth conditions). For example, the impact of oxygen and glucose conditions on constitutive yeast promoter activities was characterized to permit design for these culture conditions [4].

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Known variation in tRNA availabilities among hosts has been used to reduce the host-dependence of protein expression via codon optimization [5, 6]. Alternatively, insulated elements have been developed that behave robustly in varying contexts. For example, researchers have developed an insulated constitutive bacterial promoter library with relative protein production rates that span two orders of magnitude and are independent of the coding sequence of the expressed protein [7]. New insulating elements that use RNA processing to reduce the context dependence of genes in multi-gene operons have also been introduced [8].

Quantitative modeling and characterization of control elements have enabled researchers to create larger libraries of elements that exhibit predictable behaviors when integrated into gene expression devices. For example, a thermodynamic model of bacterial translation initiation was developed and used to forward design synthetic RBSs with a 47% chance of exhibiting protein expression levels within 2.3-fold of the desired level [9]. In addition, libraries of novel gene control elements have been developed using evolutionary and screening strategies. For example, a set of Rnt1p-cleavable hairpins provides post-transcriptional tuning of protein expression levels ranging from 8-84% of a control construct without a hairpin [10]. *In vivo* scaffolds are another set of synthetic control elements and act post-translationally to improve pathway flux by spatially co-localizing enzymes to RNA [11], DNA [12], protein [13, 14], cell surface [15], or a specific organelle [16].

Along with the diversification and insulation of genetic control elements have come faster, more reliable methods to construct biosynthetic pathways. Notably, an eight-gene biosynthetic pathway was assembled into a shuttle vector or yeast artificial chromosome in a single transformation with over 50% efficiency (Figure 1a) [17]. Pathways can also be integrated iteratively, which may increase the accessible library size for testing variants in a multi-gene pathway [18]. The construction and transplantation of a chemically synthesized bacterial genome showcased the cumulative advances of *in vitro* enzyme-mediated assembly and *in vivo* transformation-associated-recombination in yeast [19]. Similar techniques have been used to replace chromosome arms in yeast with circular or linear synthetic versions [20]. In *E. coli*, researchers have developed “recombineering” methods that use phage proteins to facilitate recombination-based genetic engineering. Rec-mediated recombineering was developed for efficient recombination between linear PCR products and linearized plasmids, which complements efficient lambda phage Red-mediated recombination between linear PCR products and circular plasmids [21].

Improved tools for *de novo* pathway identification

Engineered biosynthetic pathways were once painstakingly pieced together from a single organism’s cDNA to mimic natural biosynthesis strategies, and optimization of host platforms involved serial knockouts or overexpression of targeted genes. However, modern bioinformatic tools allow rapid mining of huge sequence repositories for functions of interest and even prediction of multiple possible pathways to produce a desired small molecule (Figure 1a). Databases such as BioCyc [22], Kyoto Encyclopedia of Genes and Genomes (KEGG) [23], Rhea [24], and Braunschweig Enzyme Database (BRENDA) [25] facilitate search and display of metabolic pathways. These databases and others are populated by proteins whose discovery is accelerated by functional prediction algorithms, e.g., global biochemical reconstruction using sampling (GLOBUS), that take a systems biology approach to adjusting functional predictions generated by homology and secondary structure [26]. Genome-scale metabolic models of several industrially useful host organisms are available and reviewed elsewhere [27]. These stoichiometric models are used in computational predictions of routes from central metabolites to a product of interest, implementing a variety of rules for reaction qualifications (e.g., known enzymes,

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metabolites, or reaction chemistries) and scoring schemes (e.g., route length, number of enzymes known, complexity of transformations, flux balance analysis) to rank possible pathways [28]. For example, researchers used in-house pathway prediction and ranking software with a genome-scale *E. coli* model to identify two possible pathways to the non-natural product 1,4-butanediol [29].

In a creative use of genomic information, an alkane-producing operon was discovered via subtractive genomic analysis of 11 cyanobacteria strains and used in *E. coli* to produce a mixture of alkanes and alkenes [30]. Enzymes variants can be rationally selected when enzymes with similar functions from many organisms are available. In the reverse engineering of the β- oxidation cycle for production of *n*-butanol, enzymes were chosen based on kinetic parameters and co-factor specificity [31, 32]. The combination of genomic information and library-based cloning strategies is a rapid way to obtain rare enzymatic activities. In one example, a 30 kb *Vibrio splendidus* genome fragment, suspected by homology to encode transporters needed for alginate utilization, was cloned into *E. coli* by screening a genomic fosmid library [33].

Tools for molecular diversity generation and screening

Directed evolution is a powerful method for optimizing biosynthetic pathways and requires molecular tools for generating and screening diversity (Figure 1c). Tools for generating diversity at the single-gene level, such as error-prone PCR, DNA shuffling, saturation mutagenesis, and site-directed mutagenesis, have been used to generate enzyme libraries of varying size for some time. Screening for small molecule production depends on physical characteristics of the substrates, cofactors, products, or strains. Screens employ selection, growth, colorimetric, fluorescent, or UV readouts in a high-throughput format; or direct quantification by a separation method coupled to a detection method, like LC-MS, in lower-throughput formats. Recent work to engineer *E. coli* for increased levopimaradiene production provides examples of both small and large library generation and screening [34]. One enzyme in the pathway, levopmaradiene synthase, was iteratively subjected to site-directed and single-site saturation mutagenesis at amino acid positions selected from a structural homology model, and resulting changes in productivity were analyzed by GC-MS. A second enzyme, geranylgeranyl diphosphate synthase, was subjected to error-prone PCR, expressed in the context of a lycopene-producing strain, and then screened visually for production of the red pigment characteristic of lycopene. Together, these two optimized enzymes, along with higher expression of four upstream endogenous enzymes, increased levopimaradiene production from 0.15 mg/L to 700 mg/L [34].

Advances in DNA synthesis support the design of synthetic oligonucleotides and genes; thus smaller libraries with defined variation and a higher fraction of functional proteins can be built, permitting lower-throughput, higher-sensitivity screens (Figure 1c). Several commercial DNA synthesis companies offer tailored synthetic libraries, including specified amino acid frequencies at each site, truncations of varied length, mixed-and-matched domains from similar proteins, and specific frequencies of random mutations in all or a subset of amino acid sites. Structure or structural homology, phylogeny, and known amino acid sequence-function relationships often inform the design of these tailored libraries. For example, a computational structure-guided recombination method was used to divide cellulases into domains, and a subset of all possible chimeras was synthesized to sample the effect of each domain on thermostability and activity [35]. Linear regression analysis and machine learning were then applied to the results from the first chimera library and used to design a second small synthetic library, which yielded three variants with similar activities to the parent and greatly enhanced thermostability.

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Several tools for generating directed diversity at the genome level have been developed. Some genome alteration methods build on recombineering *E. coli*. For example, a process termed "multiplex automated genome engineering" (MAGE) introduces changes mediated by lambda phage Red and directed by synthetic oligonucleotides to generate up to 15 billion genetically distinct *E. coli* cell populations in three days of automated transformation cycles [36]. Since MAGE is most efficient with a pool of ~10 oligonucleotides, a hierarchical conjugation assembly genome engineering (CAGE) method was developed to combine altered portions of the genome into a single strain using *E. coli* donor-receptor strains engineered for site-specific recombination [37]. A trackable multiplex recombineering (TRMR) method was developed to introduce thousands of targeted and barcoded changes into the bacterial genome such that fitness phenotypes can be mapped back to the specific change in the genome [38]. This approach was recently used with MAGE to combinatorially alter the ribosome binding sequences of thousands of genes and map resulting growth phenotypes to their accompanying genomic changes [39]. In yeast, homologous recombination has been used for generating diversity via transformation with libraries of synthetic oligonucleotides flanked by homology regions [40]. Additionally, homologous recombination has been combined with an inducible double-stranded break system and sexual reproduction to generate rounds of diversity *in vivo* by mating rather than by transformation [41].

Optimal enzymes or control elements for a biosynthetic pathway can only be identified through appropriate screens; thus, generalizable, high-throughput screens are highly desirable. For example, enzyme activity can be assayed indirectly by monitoring consumption or production of a UV-active cofactor like NAD(P)H or using either a general colorimetric assay for pH or an organic functional group. However, these assays can be difficult to conduct *in vivo* and may not have the necessary sensitivity and signal-to-noise ratio to detect small changes in enzyme function. Recently, a screening strategy was described that combines yeast display of an enzyme library, chemoenzymatic conjugation of one substrate to the cell surface, incubation with a second substrate with an affinity handle, fluorescent staining of or conjugation to the affinity handle, and fluorescence-activated cell sorting (FACS) to select cells expressing enzymes capable of forming bonds between the two substrates [42]. Although this method is not dependent on physical properties of the substrates or product, it does require that both substrates be amenable to chemical conjugation and that the enzyme being selected tolerate surface display and have a sufficiently flexible active site. In an alternative approach, an *in vivo* RNA-based biosensor couples intracellular small-molecule product concentrations to levels of a genetically encoded reporter like GFP, such that large enzyme or pathway libraries can be rapidly screened by FACS [43]. This method depends on selection of an appropriate RNA aptamer to the small molecule target of interest and integration of the aptamer into the RNA biosensor platform, rather than on any innate property of the substrate, product, or enzyme.

Small molecule biosensors that can be linked to gene expression are particularly powerful in the context of the recently developed *in vivo* phage-assisted continuous evolution (PACE) [44]. In PACE, host *E. coli* cells express two plasmids, one for continuous mutagenesis and one on which the expression of a protein necessary for phage survival is linked to a desired protein activity. The host *E. coli* is infected by selection phage that encode the protein library, and the culture is maintained in a turbidostat such that phage that encode protein library members that activate the expression of the phage-survival protein are enriched over time.

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Systems biology analysis for host-level optimization

As tools and methods for identifying, building, and optimizing heterologous metabolic pathways have grown in number, the need to understand host metabolism at a systems level to support further optimization has emerged. To meet this need, systems biology has developed techniques to support "-ome-level" interrogation of cellular behavior and quantitative models for analysis of whole-cell metabolic networks (Figure 1d) [45-47]. The use of systems-level measurements has expanded researchers' abilities to identify gene targets to improve engineered strains. For example, transcriptome profiling has been used to identify sources of stress in yeast strains harboring evolved P450 monooxygenases. By analyzing the global transcriptional response across a series of evolved enzyme variants, researchers identified heme depletion as the major limiting factor for optimized monooxygenase activity at high expression. Subsequent overexpression of cellular machinery for producing heme increased the productivity of the highest activity evolved variant by 2.3-fold [48]. In another study, global transcript, metabolite, and genotype measurements were used to identify traits associated with higher yeast growth rates on galactose, an industrially relevant sugar disfavored by native yeast metabolism. Researchers compared two previously engineered strains and three newly evolved strains against a parent strain and identified specific favorable mutations that arose in gene targets unpredictably related to carbohydrate sensing and catabolism [49].

Improvements in stoichiometric genome-scale metabolic networks with constraint-based models like flux balance analysis (FBA) or minimization of metabolic adjustment (MOMA) have enabled several recent model-guided strain optimization efforts, in which predicted modifications of host metabolism improved product yields (Figure 1d) [50]. For example, OptGene, a genetic search algorithm for non-linear optimization [51], and MOMA were used to identify knockout targets within a stoichiometric model of *S. cerevisiae* that would lead to increased sesquiterpene production [52, 53]. Deletion of the predicted target glutamate dehydrogenase *GDH1* and overexpression of the NADH-dependent glutamate dehydrogenase gene *GDH2* to repair the consequent growth defect, resulted in a strain with nearly triple the total sesquiterpene titer [53]. Using similar computational methods, researchers simulated the effect of gene knockouts on the relationship between growth rate and polylactic acid production rate, identifying three knockout targets that were combined with two rationally selected overexpression targets to increase overall polymer accumulation by 3.7-fold [54]. An engineered strain of *E. coli* for the production of 1,4-butanediol (BDO) was improved by the introduction of targeted changes to the host genome guided by a whole-cell metabolic model [29, 55, 56]. The changes made to host metabolism included knockouts of key enzymes, deletion of a global regulator, and a point mutation in an enzyme to destroy the allosteric inhibition of the native citrate synthase, leading to over 95% of carbon flux being directed to the BDO pathway as measured by 13C labeling [29].

Molecular control elements for dynamic pathway regulation

Taking a cue from natural regulatory networks, researchers have begun designing dynamic regulation for engineered biosynthetic pathways (Figure 1b) [57, 58]. For example, a theoretical analysis detailed parameter constraints necessary to build a biological proportional-integral control network capable of perfect adaptation and modeled the effects of these parameters in a two-promoter gene network [59]. An *in silico* model of dynamic control in a biofuel production pathway concluded that efflux pumps under control of a biofuel-responsive promoter would limit toxicity [60]. However, implementation of these *in silico* designs is currently limited by availability and tunability of the molecular components required to build dynamic controllers.

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Genetically encoded sensors developed for selection and screening can potentially be coupled to activators to create synthetic molecular dynamic controllers [61]. While conceptually simple, building such a device can be practically challenging and demands parameter tuning to obtain desired behavior [62]. For example, nine distinct parameters were involved in the design of static ribozyme-regulated expression devices [63]. Dynamic controllers sense and process a molecular input and generate a gene-regulatory output. Open-loop controllers sense an external molecular input, such as the IPTG supplied to an IPTG-inducible promoter, while closed-loop controllers sense a molecular input associated with the pathway of interest, such as an intermediate in an engineered biosynthetic pathway [61]. In a recent implementation of open-loop control, researchers used the glucose-responsive promoters *HXT1* and *HXT2* to control the expression level of squalene synthase, *ERG9*, resulting in a 2-fold increase in α-santalene production in yeast, which was improved by further host metabolic engineering and bioprocess optimization strategies [64]. In addition to concentrations of small molecules, open-loop controllers can respond to autoinduction by cellular quorum sensing. By engineering the native quorum sensing regulon to initiate the expression of T7 polymerase, researchers demonstrated an autoinduction "switch" that serves as a late stage *E. coli* heterologous protein expression system [65].

Because closed-loop control requires response to a signal associated with the biosynthetic pathway, it can be more complex to engineer. The first example of a closed-loop metabolic controller was a synthetic regulon built using parts of the acetyl phosphate-responsive promoter *glnAP2* applied to the control of enzyme expression levels in the lycopene biosynthesis pathway. As metabolic flux to acetyl phosphate competes with that to lycopene, this controller served to divert flux to the lycopene pathway, increasing productivity 3-fold [66]. In a recent example, researchers built a dynamic closed-loop controller from the natural fatty-acid sensing transcription factor FadR, which binds to and represses a recombinant promoter [67]. High cellular concentrations of fatty acids lead to increased pools of acyl-CoA that bind FadR, which then releases its DNA binding region and allows transcription from the engineered promoter. This promoter was used to control the expression of *pdc* and *adhB* for ethanol production, *fadD* for fatty acyl-CoA production, and *atfA* for fatty acid ethyl ester (FAEE) production. The dynamic control system effectively prevents the build-up of ethanol, a toxic pathway intermediate, and over-production of acyl-CoA, which consumes fatty acids needed for other processes. FAEE yield increased by 3-fold to 28% of the theoretical maximum under the dynamic control scheme. These systems demonstrate the promise of dynamic control strategies for engineered biosynthetic pathways, and show that repurposing of native systems can be a useful starting point for building dynamic controllers [67].

Conclusion

Enhancing our ability to engineer controlled, multi-enzyme biosynthetic pathways in whole-cell hosts is essential to expanding the spectrum of fine and bulk products that can be made with chemical biotechnology. Molecular tools for pathway identification, rapid and precise pathway construction, directed evolution for component optimization, systems-level models and measurements for host optimization, and dynamic pathway control advance these efforts. While a convergent set of tools and methodologies that allow researchers to build pathways to synthesize molecules of interest with ease is desired, developing generally applicable tools is an overarching challenge in chemical biotechnology. As shown in Table 1, solutions to improve pathway productivity are often specific to the host organism, the small molecule product, or even the particular strain. Much work remains to gain the predictive understanding necessary to build a biosynthetic pathway "off-the-shelf" and will involve amassing particular solutions to find generalizable patterns for strategic construction

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and optimization as well as developing generalizable, robust biological components and system composition methods.

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Highlights

- Novel molecular tools advance the productivity of engineered multi-enzyme biochemical pathways in live cells.
- These tools enable precise, rapid pathway construction and static and dynamic control of enzyme expression levels.
- Molecular tools facilitate enzyme or pathway optimization via directed evolution and systems biology approaches.
- The generalizability of each tool depends on its function, context dependence, and host requirements.

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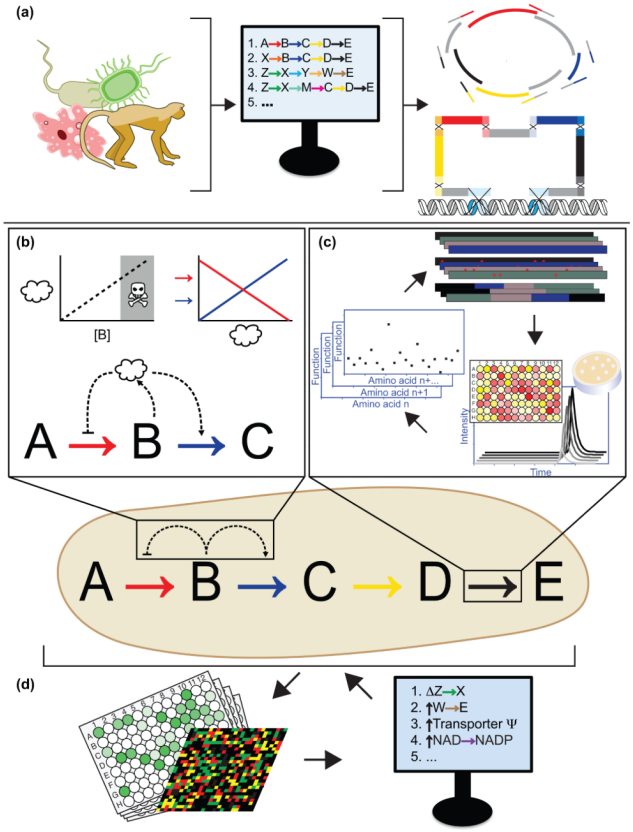

Figure 1.

Molecular tools to advance engineering of multi-enzyme biochemical pathways for chemical biotechnology. (a) Genetic and genomic databases are used with predictive algorithms to design pathways, which are then genetically constructed *in vitro* and/or *in vivo*. (b) Dynamic control elements allow enzyme expression levels to vary in response to small molecule concentrations. (c) Rounds of diversity generation and screening, informed by machine learning and design of experiment algorithms, generate optimized enzyme variants. (d) Whole cell read-outs provide data for systems level analysis and prediction of specific changes to enable global phenotypic improvements.

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Table 1

Generalizability of selected molecular tools for chemical biotechnology.

Role	Molecular tool	Host cell(s)	Generalizability	References
Expression tuning	Constitutive promoters	*S. cerevisiae*	Applications in yeast	[4]
Expression tuning and robustness	Codon optimization	Any	Any	[5, 6]
Expression robustness	Insulated bacterial promoters	*E. coli*	Any bacterial system where a 160 bp promoter is acceptable and elements in the 5'-UTR are not needed	[7]
Expression robustness	Clustered regularly interspaced short palindromic repeat (CRISPR) RNA processing	*E. coli, B. subtilis, S. cerevisiae*	Any organism in which the Csy4-based processing platform can be functionally expressed	[8]
Expression tuning	Synthetic ribosome binding sites	*E. coli*	Applications in *E. coli*	[9]
Expression tuning	RNA control modules based on Rnt1p hairpins	*S. cerevisiae*	Applications in yeast	[10]
Enzyme scaffolding	RNA-enzyme assemblies	*E. coli*	Applications in prokaryotes in which enzymes are amenable to tagging with aptamer binding domains	[11]
Enzyme scaffolding	DNA-enzyme assemblies	*E. coli*	Applications in prokaryotes in which enzymes are amenable to tagging with zinc-finger proteins	[12]
Enzyme scaffolding	Protein scaffold-enzyme assemblies	*E. coli, S. cerevisiae*	Applications in which enzymes are amenable to tagging with protein scaffold binding domains	[13, 15, 68, 69]
Enzyme scaffolding	Protein microcompart-ments	*E. coli*	Potential applications in prokaryotes for enzymes that are amenable to tagging with binding domains or localization tags that direct them to protein microcompartments	[70-73]
Enzyme scaffolding	Host-heterologous protein fusions for localization	*S. cerevisiae* (in this example)	Any application in which a suitable host localization protein can be identified and a functional host protein-heterologous enzyme chimera can be generated	[14]
Enzyme scaffolding	Organelle-targeting tags	*S. cerevisiae*	Applications in eukaryotes in which an appropriate organelle tag is available and the enzyme(s) is amenable to fusion	[16]
Genetic assembly	One step homologous recombinatio-nbased plasmid assembly or integration	*S. cerevisiae*	Applications requiring assembly of 8-10 DNA segments in yeast	[17]
Genetic assembly	Iterative homologous recombination-based integration	*S. cerevisiae*	Applications in yeast in which one-at-a-time integration and selection marker rescue is desired, such as pathway library construction	[18]
Genetic assembly	Combined *in vitro* and *in vivo* assembly for genome-scale constructs	*S. cerevisiae*	Applications requiring assembly of multi kB-MBconstructs in yeast	[19, 74]

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Role	Molecular tool	Host cell(s)	Generalizability	References
Genetic assembly	Construction of synthetic chromosome arms and replacement of endogenous arms	*S. cerevisiae*	Applications in yeast in which the incorporation of large/many synthetic fragments and/or deletion of many native elements for stability is desired	[20]
Genetic assembly	Recombination of linear fragments	*E. coli*	Applications in bacteria	[21]
Genome scale diversity generation	Multiplex automated genome engineering	*E. coli*	Applications in bacteria where genome-scale diversity generation is useful for optimization and a suitable screening method is available to evaluate the resulting large library	[36]
Genome scale diversity generation	Conjugation assembly genome engineering	*E. coli*	Applications in bacteria where the combination of defined genomic fragments from multiple lineages into a single genome is desired	[37]
Genome scale diversity generation	Continuous recombination	*E. coli*	Applications in bacteria where continuous recombination is useful for optimization (e.g. by combining multiple lineages) and a suitable screening method is available to evaluate the large library generated	[75]
Genome scale diversity generation	Homologous recombination of synthetic oligonucleotide libraries	*S. cerevisiae*	Applications in yeast	[40]
Genome scale diversity generation	Inducible doublestranded break and sexual reproduction	*S. cerevisiae*	Applications in yeast where desired changes can be obtained in a manageable number of manual rounds	[41]
Diversity screening	Yeast 3-hybrid chemical complementation to screen for bond-forming enzymes	*S. cerevisiae*	Applications in yeast where enzyme tolerates surface display and two substrates between which a covalent bond is formed are amenable to chemical conjugation	[42]
Diversity screening	Small moleculeresponsive synthetic RNA switch	*S. cerevisiae*	Applications where RNA that binds the molecule is available or can be selected and incorporated into a self-cleaving genetic device	[43]
Genome scale diversity generation and screening	Phage-assisted continuous evolution	*E. coli*	Applications in bacteria where the activity of the enzyme to be evolved can be linked to gene expression	[44]
Whole cell gene expression measurement	DNA microarray measurement of global transcriptional response to heterologous protein expression	*S. cerevisiae*	Applications in yeast where host metabolism or stress response is limiting to strain productivity	[48]
Model-guided host optimization	Target gene knockouts and promoter substitutions based on model prediction	*S. cerevisiae*	Applications in yeast where host metabolism is limiting to strain productivity	[53]
Model-guided host optimization	Target gene knockouts and gene overexpression based on model prediction	*E. coli*	Applications in *E. coli* where host metabolism is limiting to strain productivity	[54]

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Role	Molecular tool	Host cell(s)	Generalizability	References
Dynamic control of gene expression	Glucose responsive promoter	*S. cerevisiae*	Applications in yeast where linking enzyme expression to glucose levels improves productivity	[64]
Dynamic control of gene expression	Rewired quorum sensing regulon	*E. coli*	Applications in *E. coli* where cell-density dependent expression of proteins is desired	[65]
Dynamic control of gene expression	Fatty acid responsive transcriptional regulator	*E. coli*	Applications in bacteria where gene expression tied to cellular free fatty acid pools is desired and cross-talk between the transcriptional regulator and native transcriptional control is not limiting	[67]