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Technology development for natural product biosynthesis in *Saccharomyces cerevisiae*

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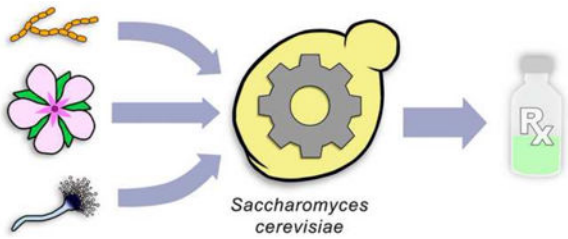
Abstract

The explosion of genomic sequence data and the significant advancements in synthetic biology have led to the development of new technologies for natural products discovery and production. Using powerful genetic tools, the yeast *Saccharomyces cerevisiae* has been engineered as a production host for natural product pathways from bacterial, fungal, and plant species. With an expanding library of characterized genetic parts, biosynthetic pathways can be refactored for optimized expression in yeast. New engineering strategies have enabled the increased production of valuable secondary metabolites by tuning metabolic pathways. Improvements in high-throughput screening methods have facilitated the rapid identification of variants with improved biosynthetic capabilities. In this review, we focus on the molecular tools and engineering strategies that have recently empowered heterologous natural product biosynthesis.

Graphical abstract



Introduction

Natural products play an indispensable role as pharmaceuticals, specialty chemicals, and industrially relevant compounds. The recent surge of genomics data has revitalized the

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discovery and characterization of enzymatic machinery used by plants, fungi, and bacteria for natural product biosynthesis. Meanwhile, the development of new genetic tools for engineering the yeast *Saccharomyces cerevisiae* has fueled efforts to refactor biosynthetic pathways for heterologous production of valuable compounds (Fig. 1). In contrast to many native production hosts, yeast is fermentable, genetically tractable, and generally recognized as safe. Furthermore, many natural product biosynthesis enzymes have been successfully reconstituted in *S. cerevisiae* that show poor activity when expressed in the model prokaryote *Escherichia coli* [1]. Strategies drawing from genomics, synthetic biology, and metabolic engineering have been applied to refactor biosynthetic pathways and optimize production of secondary metabolites. In the past several years, numerous pioneering accomplishments in the field have been realized, including reconstitution of complex pathways [2] and industrial scale-up of engineered systems [3]. The development of tools and strategies employed to engineer yeast for natural product production will be discussed.

Refactoring biosynthetic gene clusters

Refactoring biosynthetic gene clusters (BGCs) involves restructuring the genetic elements of the pathway with characterized regulatory parts for expression in the native producer or a heterologous host. The Voigt group established the methodology for biosynthetic pathway refactoring when they rebuilt the entire 23.5 kb nitrogen fixation gene cluster from *Klebsiella oxytoca* with synthetic parts for de-regulated expression in the native host [4]. During pathway refactoring for heterologous expression in *S. cerevisiae*, each gene in a BGC is either synthesized or amplified from genomic DNA or cDNA. Promoters and terminators are cloned upstream and downstream of each coding region to generate expression cassettes, which are assembled and introduced into the desired platform strain (Fig. 2).

Preparing pathway genes for heterologous expression

Pathway refactoring of gene clusters identified from eukaryotic genomic DNA often requires the removal of non-coding intron sequences. Fungal species, such as *Aspergillus*, contain many introns [5] and individual genes involved in natural products pathways frequently contain as many as 5-9 introns [6]. Although there are several methods for successful *in silico* intron prediction from genome sequences [7], even one improperly annotated intron can derail pathway characterization efforts due to incorrect protein translation. Intron-less cDNA can be generated from active gene clusters, but silent gene clusters cannot be interrogated by this approach. Further, the native spliceosome of *S. cerevisiae* does not have the capability to remove introns from distant fungi [8]. Through the mutation of specific yeast splicing factors, we have enabled the recognition of intron sequence motifs from *Aspergillus fumigatus* (unpublished data). With the continued rise in genomic sequence data, it will become increasingly important to develop new computational and experimental tools to solve the intron problem.

Despite the difficulties caused by heterologous introns in the original pathway sequence, introns native to *S. cerevisiae* have recently been added to the synthetic biology toolbox as predictable regulators of gene expression levels. Yofe *et al.* generated a gene expression

library consisting of 240 unique yeast strains, each expressing a yellow fluorescent protein (YFP) gene interrupted by a natural *S. cerevisiae* intron [9]. By quantifying the fluorescence, introns were shown to reliably reduce reporter gene expression. Incorporating *S. cerevisiae* introns into gene assembly strategies would be useful for tuning optimal expression ratios of the different pathway enzymes, especially if promoter choices are limited due to the need for specific pathway regulation.

Either through gene synthesis or site-directed mutagenesis, sequences can be codon-optimized for improved translational efficiency in *S. cerevisiae*. Codon-optimizing two genes in the carotenoid pathway (*crtI* and *crtYB*) improved beta-carotene production in yeast by 200% [10]. However, as recent work by Lanza *et al*. has shown, genes designed by traditional algorithms do not always yield higher expression than the wild type gene [11]. The authors proposed a condition-specific approach to determine optimal codon usage under a given growth condition instead of relying on the frequency of codon usage in the entire genome. The catechol 1,2-dioxygenase gene from *Acinetobacter baylyi* was optimized for production at stationary phase and had 2.6-fold higher catalytic activity than the wild type and 2.9-fold higher activity than a commercially optimized variant.

Regulatory DNA sequences control the strength of gene expression

Each coding region must be flanked by a promoter and a terminator, which are regulatory DNA sequences that influence the frequency of gene transcription and the stability of the transcripts, respectively. Promoters, classified as constitutive or regulated, are *cis*-acting regulatory elements upstream of a coding sequence where transcription factors bind and recruit the RNA polymerase in order to initiate transcription. The report by Sun *et al*. detailed the characterization of 14 constitutive promoters via expression of green fluorescent protein (GFP) under varied glucose and oxygen conditions [12]. Promoters of high and medium strength were used in the refactoring of xylan degradation and zeaxanthin biosynthesis pathways and the resultant yeast strain produced 0.74 mg/L of zeaxanthin.

To avoid the generation of toxic products and metabolic stress during heterologous expression, cell growth can be decoupled from the production phase using regulated promoters, which are inducible or repressible under different physiological conditions. For example, a modified GAL induction system, repressed by glucose and induced by galactose, was used successfully in the production of artemisinic acid at 25 g/L in *S. cerevisiae* [3]. Another glucose repressible promoter, ADH2p, induces strong transcription once glucose is depleted and has been shown to provide higher expression levels than the GAL promoter [13]. The ADH2 promoter has proven highly useful for the heterologous expression of biosynthetic genes and recently enabled the discovery of new fungal indole diterpenes [14]. A library of ADH2-like promoters would prove beneficial for pathway refactoring and characterization of new biosynthetic gene clusters.

Unfortunately, yeast promoters tend to be hundreds of base pairs in length, which compounds the difficulties for rapid pathway refactoring. Recent work by Redden and Alper has focused on the construction of short synthetic promoters [15]. The authors combined core promoter elements with upstream activation sequences to generate minimal promoters.

They achieved high levels of both inducible and constitutive expression with an up to 80% reduction in the size of the promoter.

The terminator sequences, downstream of the coding region, influence the mRNA half-life. Yamanishi *et al*. evaluated 5302 terminator regions by quantifying the level of GFP expressed under the control of a strong promoter [16]. Work by Curran *et al*. focused on characterizing the activity of over 30 terminator regions for metabolic engineering applications in yeast [17]. Terminators were coupled to promoters with a variety of strengths and a yellow fluorescent protein was expressed and quantified with FACS. The Alper group also systematically developed short (35-70 bp) synthetic terminators and proved they function as effectively as native terminators for the expression of the heterologous proteins involved in the biosynthesis of itaconic acid [18].

Episomal expression and plasmid assembly techniques

Refactored genes can be assembled into plasmids or integrated into the genome for expression in yeast. Episomal expression is an effective strategy as there are a variety of efficient methods for plasmid assembly and high copy numbers are accessible with few cloning steps. During plasmid construction, the identity of the selection marker and origin of replication must be decided upon depending on the genotype of the yeast strain used and desired copy number of the plasmid. Prototrophic markers and markers conferring drug resistance are commonly used selection markers [19]. However, the identity of the selection marker can negatively impact the growth rates of yeast and the metabolite production levels, particularly in haploid strains [20]. Plasmid copy number is controlled by two frequently used origins: low copy CEN/ARS and high copy 2 μ. Low copy plasmids have been found to generate reliable expression patterns, while high copy plasmids can display more variable expression [21]. The identity of the gene being expressed can also influence which origin to select. In a recent report by Trenchard and Smolke, it was found that low copy expression of plant P450s yielded higher production levels of the target compound cheilanthifoline [22]. High copy expression of the endomembrane-localized P450s induced a stress response in the endoplasmic reticulum, causing membrane proliferation, and ultimately lowered cheilanthifoline biosynthesis.

There have been many recent advances for the *in vitro* and *in vivo* assembly of DNA parts. Gibson assembly is a sequence independent, one-pot method for assembling multiple overlapping DNA fragments through the combined activities of an exonuclease, polymerase, and ligase [23]. Golden Gate method, which is sequence dependent, utilizes type II restriction enzymes to efficiently combine modular parts in a one-pot reaction [24] and an extension of this technology has recently been adapted specifically for yeast assemblies [21]. The methodologies based on Golden Gate do not require downstream sequencing of assembled constructs as point mutations cannot arise once the individual components have been generated. The homologous recombination machinery of yeast can also be utilized for the assembly of plasmids *in vivo*, requiring only one transformation step and as few as 29 nucleotides of overlapping sequence between DNA fragments [25]. Yeast homologous recombination has been used to refactor silent and orphan gene clusters, such as in the discovery of the antibiotic taromycin A from the marine actinomycete *Saccharomonospora*

[26], and potent indolotryptoline antiproliferative agents, lazarimides A and B, from environmentally-derived DNA [27].

Genomic expression and gene integration technologies

Integration of heterologous genes has unique advantages over episomal expression, particularly for metabolic engineering applications. Genes integrated through homologous recombination are stably maintained in cell populations without the need for selection pressure, although simultaneous integration of a marker is necessary to select for integrants. If higher copy number is desired, multiple copies of pathway genes can be sequentially targeted to multiple locations in the genome or integrated using the repeating delta sites [28]. For this purpose, the expression levels of many loci have been characterized using *lacZ* and luciferase reporters [29], [30]. In a recent report by Brown *et al.*, the plant natural product strictosidine was detected after a rate-limiting enzyme in the pathway was integrated multiple times into the genome and expressed from a high copy plasmid [31].

Recent advancements in integration technology revolve around the induction of a double-strand break (DSB) in the DNA to greatly improve the efficiency of integration. The homing endonuclease I-SceI was used to introduce a DSB at a target locus and eight overlapping fragments, seven genes and one selection marker, were efficiently integrated simultaneously [32]. Another robust method for the construction of multigene pathways is Reiterative Recombination [33]. Two endonucleases, SceI and HO, are used iteratively in conjunction with recyclable markers to sequentially assembly DNA fragments into a single locus with high efficiencies. Despite these elegant advances, the development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated systems (Cas) has ushered in a new era of efficient marker-free genome editing, promising greater flexibility and utility than previous DSB technologies. First adapted for utilization in yeast by DiCarlo *et al.* [34], CRISPR/Cas9 has proven to be a powerful tool for rapid pathway construction. Mans *et al.* demonstrated the simultaneous integration of 6 genes, composing the *Enterococcus faecalis* pyruvate dehydrogenase complex, at a single locus combined with a separate gene deletion to construct an acetyl-CoA overproducing strain [35]. The potential of multigene pathway assembly using CRISPR/Cas9 was further explored in a report by Jakočiūnas *et al.* [36]. A total of 15 DNA parts (3 expression cassettes) were integrated into three separate loci without selection and with an efficiency of 31%. By programming CRISPR/Cas9 to cut at the repeating delta sites, 18 genomic copies of a combined xylose utilization and (*R,R*)-2,3-butanediol production pathway were integrated in one transformation step [37]. The ability to assemble gene clusters at any genomic location through the combined use of DSB technologies and yeast homologous recombination will prove invaluable for the continued production of natural products in yeast.

Strategies to optimize production of secondary metabolites

Genetic refactoring plays a vital role in the initial reconstitution of heterologous biosynthetic pathways. However, the power of yeast as an efficient microbial production factory can only be realized after secondary metabolite production is extensively optimized. Yeast strains producing low levels of natural products thus provide important platforms for exploring

strategies that may be applied to increase rates, titers, and yields. In recent years, a variety of tools have been reported which have allowed engineers to increase small molecule production many fold. These approaches, shown in Fig. 3, typically involve tuning endogenous yeast metabolism, engineering components of the heterologous pathway, and employing high throughput screening methods.

Redirecting the endogenous yeast metabolic network

Natural product biosynthesis in *S. cerevisiae* relies on tuning endogenous metabolism as a necessary step to ensure sufficient precursor supply. Many cofactors and substrates required for the biosynthesis of secondary metabolites are shared by yeast primary metabolism, which is robust and complex [38]. Furthermore, pathway intermediates can be metabolized or excreted by yeast or can exert toxic effects by disrupting normal biomolecular functions. A survey of successful metabolic engineering efforts indicates that developing platform strains producing high levels of precursors enhances production of complex secondary metabolites. Nielsen recently highlighted that a single platform strain can be adapted to produce a versatile array of different natural products [39]. Advancements in the ability to modify yeast metabolism have resulted in the development of platform strains leveraged for production of valuable compounds.

A large number of natural products including terpenes, polyketides, and sterols are derived from the primary yeast metabolite, acetyl-CoA. Acetyl-CoA plays a critical role in carbon catabolism, but can also be converted into the fatty acid precursor malonyl-CoA or shuttled into the mevalonate pathway for isoprenoid production. In order to boost cytosolic acetyl-CoA levels, Chen *et al.* employed a combined “push-pull-block” strategy to engineer the pyruvate dehydrogenase bypass. Overexpressing yeast ADH2 and ALD6, involved in the conversion of ethanol to acetate, as well as overexpression of an acetyl-CoA C-acetyltransferase resulted in enhanced production of the sesquiterpene α-santalene [40]. Lian *et al.* took a different approach to construct a platform strain by deleting four genes implicated in ethanol and glycerol production, thus redirecting flux towards acetyl-CoA [41]. Each of the aforementioned strategies also employed a non-feedback regulated mutant acetyl-CoA synthetase from *Salmonella enterica* characterized in an earlier study [42]. An impressive 37-fold increase in the acetyl-CoA/malonyl-CoA derived polyketide triacetic acid lactone was obtained through a combination of computational guided knockouts and culture attenuation [43]. Optimization of yeast metabolism for production of downstream isoprenoids via the mevalonate pathway has been extensively engineered to the point of commercial production of artemesinin [3]. In a recent report, CRISPR/Cas9 was employed to carry out multiplexed genome editing for optimization of mevalonate levels by greater than 41 fold. In addition to generating a markerless, quintuple gene knock-out in a single transformation step, genome resequencing indicated that CRISPR/Cas9 endonuclease activity resulted in no increase in off-target mutations [44].

Engineering amino acid production pathways is also of great interest for natural product biosynthesis. Amino acids can be considered value-added primary metabolites and are precursors to natural products, including benzylisoquinoline alkaloids, indole alkaloids, stilbenes, flavonoids, and non-ribosomal peptides. Several platform strains have been

established in recent years for enhanced production of amino acids and their derivatives. Introduction of feedback insensitive alleles of genes involved in amino acid biosynthesis has been employed to increase extracellular concentration of aromatic compounds 200-fold [45]. This strategy was extended to the biosynthesis of *p*-coumaric acid, an important precursor to many secondary metabolites. In conjunction with gene knockouts and expression of *Escoli* pathway analogues, Rodriguez *et al.* developed a yeast strain capable of producing nearly 2 g/L *p*-coumaric acid [46]. Qin and coworkers carried out a comprehensive, modular engineering of yeast metabolism for high-level production of the amino acid intermediate L-ornithine. Pathway rewiring of the urea cycle and TCA cycle in combination with other metabolic engineering strategies enabled a 23-fold improvement in L-ornithine titers [47].

Engineering components of the exogenous pathway

Spatial organization of pathway components has been employed to increase flux through a heterologous pathway. The outcome of this system-level engineering approach is a decrease in diffusion of metabolic intermediates from pathway enzymes and an increase in flux through the pathway via substrate channeling. DeLoache recently highlighted the unique chemical environments of eukaryotic organelles as an emerging target for heterologous pathway localization [48]. Sequestering enzymes into an area of the cell with a distinct pH, redox potential, or cofactor profile allows for optimization of the reaction environment. Farhi *et al.* employed a mitochondrial signaling peptide (COX4) in order to target plant terpene biosynthesis to the yeast mitochondria. In conjunction with other efforts to enhance flux through the mevalonate pathway, subcellular localization resulted in an 8-fold increase in valencene production and a 20-fold increase in amorphadiene production [49]. Avalos *et al.* applied a similar strategy to explore the effects of pathway compartmentalization on the production of isobutanol. Targeting the Ehrlich pathway to the mitochondria resulted in a 260% increase in isobutanol titers, a marked improvement over the 10% increase when cytoplasmic overexpression was employed [50]. An opposite effect was reported when Qin *et al.* implemented pathway re-localization as a part of their modular pathway rewiring strategy for production of the amino acid intermediate L-ornithine. When a portion of the L-ornithine biosynthetic pathway was localized to the mitochondria, titers dropped significantly, whereas re-localization of the complete pathway to the cytosol resulted in increased L-ornithine production [47].

Localizing natural product biosynthetic enzymes on engineered scaffolds has also been used to increase pathway flux. Dueber and coworkers engineered a synthetic protein scaffold in *E. coli* by generating polymers of repeated interaction domain subunits from a mammalian scaffolding protein. After tuning enzyme stoichiometry, an impressive 77-fold increase in mevalonate titers was observed [51]. This system was extended to resveratrol biosynthesis in yeast and resulted in a 5-fold improvement relative to a non-scaffolded control strain [52]. Zalatan e*t al.* employed endonuclease deficient Cas9 scaffolds to execute complex transcriptional programming rules and modulate flux through the branched violacein pathway reconstituted in yeast [53].

Another strategy to increase pathway flux is to target the activity of a specific enzyme that has been identified as a pathway bottleneck. Not surprisingly, enzymes that have been

evolutionarily optimized to function in the native context can show decreased activity when expressed in a heterologous host. If codon-optimization and enzyme overexpression fail to address bottleneck issues, protein engineering offers an attractive solution. Both rational protein design and directed evolution (discussed below in the context of Tools for high-throughput screening) have proven to be successful tools for improving specific enzyme activities. In order to efficiently produce resveratrol in yeast, Zhang *et al.* generated a fusion protein of the last two enzymes in the resveratrol biosynthetic pathway. Expression of the fusion increased bioconversion of a resveratrol precursor up to 15-fold relative to coexpression of the individual enzymes [54]. In an effort to recapitulate substrate channeling exhibited by multidomain terpene synthases, Zhou *et al.* engineered two consecutive terpene synthesis enzymes into a single protein fusion. When this strategy was coupled with the fusion of enzymes supplying pathway precursors, the production of miltiradiene increased significantly and the accumulation of byproducts decreased [55]. Fusion of heterologous enzymes with yeast enzymes has also been utilized to increase flux towards a natural product of interest. Albertsen and coworkers doubled production of the sesquiterpene patchoulol by fusing a yeast farnesyl diphosphate synthase with a patchoulol synthase of plant origin [56]. Chemler *et al.* deployed yeast homologous recombination to assemble chimeric polyketide synthase (PKS) variants capable of combinatorial biosynthesis. By shuffling catalytic domains from two bacterial PKSs, novel antibiotic analogues were generated [57]. Domain swapping also played a key role in a recent milestone in synthetic biology: the *de novo* production of opioids in yeast. Galanie and coworkers identified an N-terminal anchoring domain responsible for the incorrect sorting and processing of a C-C coupling P450, and replaced it with the anchoring domain from a functionally expressed P450. The resulting protein chimera demonstrated a 3-6-fold increase in substrate conversion relative to the wild-type enzyme [2].

Indeed, cytochrome P450s are of particular interest with regards to engineering natural product pathway proteins in yeast. Plant and fungal P450s are N-terminally anchored into the endoplasmic reticulum, and typically co-localize with an NADPH dependent reductase partner. The remarkable chemistry performed by P450s offers great opportunities along with tremendous technical hurdles for the metabolic engineer. However, the ubiquitous presence of P450s in natural product pathways has resulted in the development of strategies to engineer P450 performance in yeast including tuning P450-reductase coupling [3] and optimization of culture conditions [22]. For a more complete review of developments and challenges regarding P450-mediated metabolic engineering, see [58].

Tools for high-throughput screening

The effectiveness of pathway engineering can be greatly enhanced if a high-throughput screening method is leveraged to identify strains with improved metabolite production. Furthermore, mutagenesis based directed evolution of enzymes and strains is only possible if a robust screening method is developed which links metabolite production with a selectable phenotype. Biosensors have been successfully employed as high-throughput screening tools with which selection is imposed either in situ (survivability during culture) or ex situ (cell sorting using FACS). High-throughput screening thus allows for numerous iterations of the design, test, build strain engineering approach to be carried out in a way that is partially or

fully automated. If a natural product is pigmented or may be coupled to production of a pigment, chromophores may serve as biosensors to facilitate pathway optimization [59], [60], [61]. Bermejo and coworkers reported a more generalizable optical sensor strategy for measuring dynamic changes in metabolite levels based on Förster resonance energy transfer (FRET). This approach takes advantage of conformational changes of a protein interaction domain fused to a fluorescent protein and subsequent changes in emission spectra. Thus, FRET based biosensors are applicable to any metabolite for which a substrate recognition element can be identified [62]. Schlect *et al.* demonstrated that a multiplexed complementation assay could be used to identify small-molecule dependent growth changes in reporter strains [63]. Engineered RNA-based riboswitches have been successfully employed as both metabolite sensors and regulatory actuators. Upon binding to a ligand, riboswitches change conformation and generate an output that typically results in controlled gene expression. Michener *et al.* developed a riboswitch reporter to link metabolite levels with GFP expression and applied this system for mutagenesis based evolution of a bacterial caffeine demethylase. Iterative screening resulted in an enzyme variant with a 33-fold increased activity and a 22-fold increased product selectivity [64]. Lee and coworkers integrated a survivability output into a riboswitch circuit for automatic *in vivo* selection of metabolite overproducing variants [65].

Future Directions

Despite the many advantages of yeast as a heterologous host for natural product biosynthesis, there are still challenges that must be overcome to fully realize the potential of this organism. During pathway refactoring efforts, introns must be removed from the protein coding sequences because the native spliceosome of *S. cerevisiae* is unable to splice heterologous introns. Proteins may prove nonfunctional or exhibit low activity when expressed despite prior intron removal and codon-optimization. Due to suboptimal pathway flux or insufficient precursor supply, titers of natural products may be too low for characterization or for further engineering applications. The requirement for selection markers in well-established strain engineering strategies reduces the throughput of multiplexed genomic modifications and pathway building. [AD1]However, an ever increasing number of tools and strategies are being applied to engineer and optimize yeast to overcome these challenges and produce secondary metabolites of great value. Our ability to successfully refactor biosynthetic pathways will continue to improve with the expansion of new synthetic biology tools, development of high throughput, computationally driven engineering approaches, and the discovery of new molecular biology phenomena. In the past several decades, scientists have documented the astonishing machinery perfected by Mother Nature over millions of years. Finally, we are beginning to leverage this information to engineer microbial production factories in order to meet the global demand for valuable natural products. Ultimately, natural product biosynthesis in *S. cerevisiae* has the potential to address this demand using technology that is safe, affordable, and sustainable.

Acknowledgments

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Highlights

New tools allow rapid refactoring and assembly of gene clusters for optimized expression in yeast

DNA double-strand break technologies enable efficient integration of biosynthetic pathways

Rewiring of yeast metabolic network generates versatile platform strains

Novel strategies facilitated by synthetic biology increase natural product titers

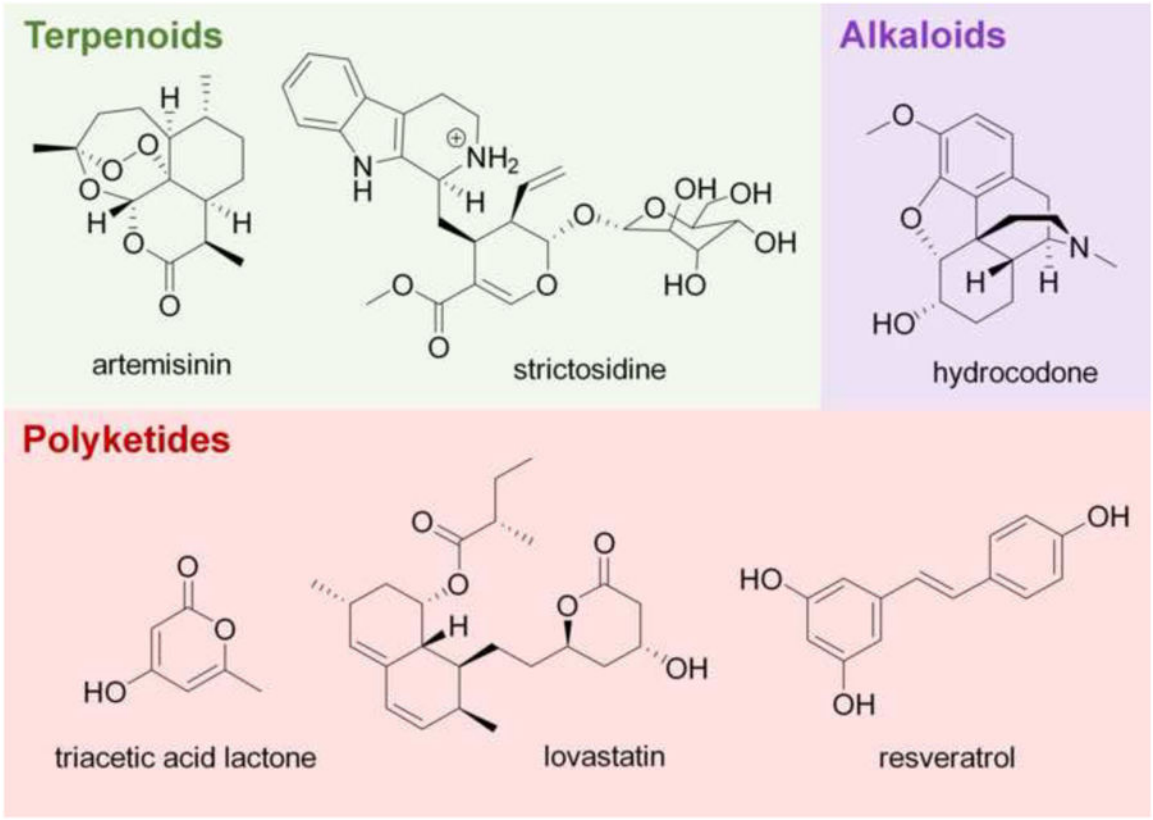

Figure 1.

S. cerevisiae has been engineered for production of a wide array of natural products.

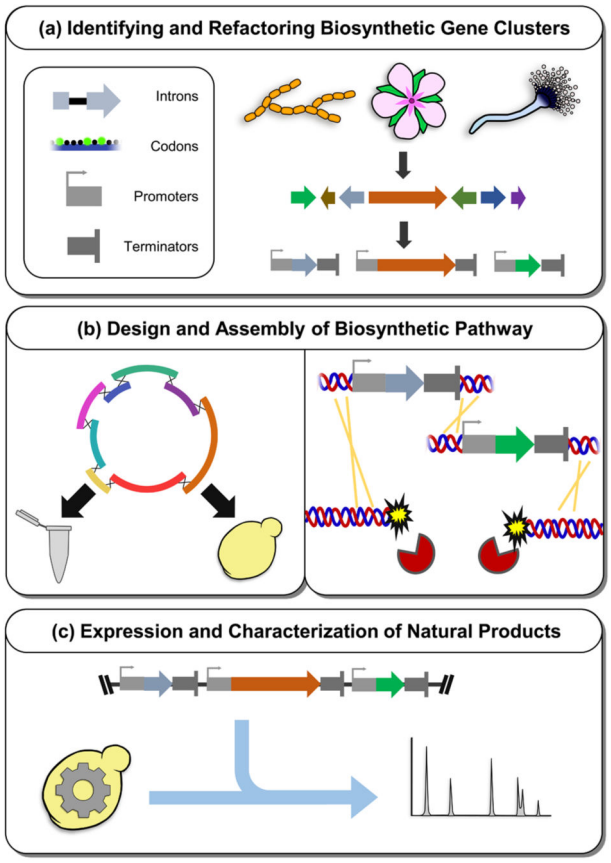

Figure 2.

(a) Overview of the process and tools used in refactoring biosynthetic gene clusters for heterologous expression in yeast. Introns are removed from eukaryotic genomic DNA sequences, codons can be optimized for improved translational efficiency in yeast, and promoters and terminators are required for tuning heterologous expression. **(b)** (Left) *In vitro* and *in vivo* DNA Assembly of plasmids for episomal expression. (Right) Multigene integration of refactored pathway genes is facilitated by DNA double-strand break technology. **(c)** Expression of the refactored biosynthetic pathway in a pre-engineered platform strain for detection and characterization of natural products.

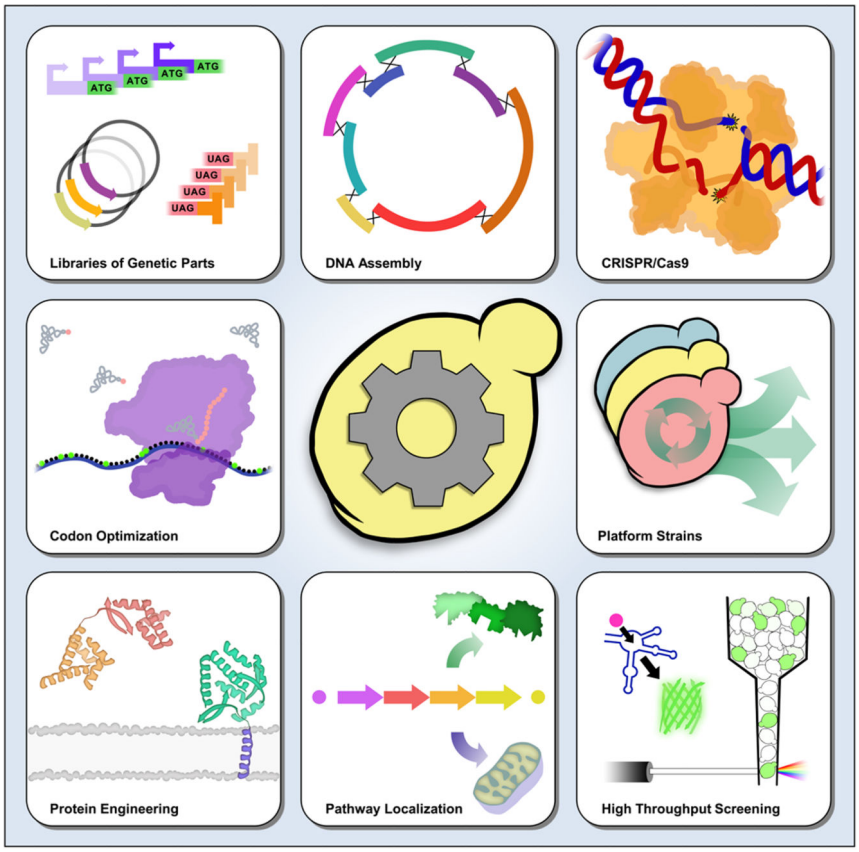

Figure 3.
An expanding engineering toolkit for natural product biosynthesis in *S. cerevisiae*.