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Frontiers of yeast metabolic engineering: diversifying beyond ethanol and *Saccharomyces*

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Microbial systems provide an attractive, renewable route to produce desired organic molecules such as fuels and chemicals. While attention within the field of metabolic engineering has mostly focused on *Escherichia coli*, yeast is a potent host and growing host for industrial products and has many outstanding, biotechnologically desirable native traits. Thus, there has been a recent shift in focus toward yeast as production hosts to replace *E. coli*. As such, products have diversified in yeast beyond simply ethanol. Additionally, nonconventional yeasts have been considered to move beyond *Saccharomyces cerevisiae*. This review highlights recent advances in metabolic engineering of yeasts for producing value-added chemical compounds including alcohols, sugar derivatives, organic acids, fats, terpenes, aromatics, and polyketides. Furthermore, we will also discuss the future direction of metabolic engineering of yeasts.

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Introduction

Fungal systems have an extensive biotechnological track record for both ethanol fermentations and enzyme production [1,2]. Yet, the past 20 years of metabolic engineering have focused rather extensively on diversifying chemical production in the bacterial system *Escherichia coli* [3•,4–6]. Many of these advances are the result of the simplicity of *E. coli* metabolism characterized by minimal central metabolic pathways and robust, yet centralized regulatory systems. However, *E. coli* is not always the ideal fermentation host due to relatively low stress tolerance [7], a lack of post-translational modifications, difficulty in expressing complex enzymes like P450s [2], and a lack of subcellular compartments. In contrast, yeasts often possess these ideal characteristics and also have favorable bioprocessing traits such as a larger cell size (thus enabling an easier separation), a lower growth temperature, lower pH and by-product tolerance [8], and a lack of potential phage contamination. Moreover, yeast mating allows for improved cellular engineering and can lead to diploids with robust growth and increased adaptation [9]. Collectively, these advantageous traits support the industrial use of yeast for chemical and fuel production. For example, the subcellular compartmentalization of yeasts allows for pathway isolation and increased fluxes of heterologous products [10,11•]. Furthermore, the yeast kingdom is quite broad and while *S. cerevisiae* is conventionally used for metabolic engineering, robust nonconventional yeasts such as *Yarrowia lipolytica* and *Pichia ciferrii* are increasingly being recognized as promising hosts for the production of unique and valuable compounds [12,13••]. Thus, interest has begun to switch from *E. coli* to yeasts as production hosts.

In this review, we will focus on the recent advancements in diversifying the value-added chemicals made by yeast (Table 1). In addition, we explore the benefits and drawbacks of using yeast as production hosts. In particular, we discuss metabolic engineering efforts to produce alcohols, sugar derivatives, organic acids, fats, terpenes, aromatics, and polyketides. We conclude with a brief evaluation of where the field stands with respect to diversifying products and expanding beyond simply *Saccharomyces*.

Value-added chemical production in yeasts

Traditional successes in yeast biotechnology surround ethanol as the product and *Saccharomyces* as the species. Contemporary metabolic engineering relies on bypassing native feedback inhibition, constructing heterologous pathways and general optimization and rewiring of metabolic flux. Thanks to these efforts, yeasts have become potent hosts for producing new chemicals such as alcohols, sugar derivatives, organic acid, fats, terpenes, aromatics, and polyketides. Many of these chemicals are linked metabolically as they branch from either acetyl-CoA or pyruvate (Figure 1). We briefly discuss each of these classes below and relate how metabolic engineering has enabled production in yeast.

Alcohols

S. cerevisiae has long served as a host organism for ethanol production both for beverages and recently, for liquid

Table 1

***Diversifying beyond ethanol and Saccharomyces*: Production of variety of value-added chemicals from different species of yeasts showing production and direct carbon source**

Chemical	Organism	Titer	Central metabolism precursor
Butanol [20]	*S. cerevisiae*	2.5 mg/L	Acetyl-CoA
Isobutanol [11•,23•]	*S. cerevisiae*	0.63 g/L	Pyruvate
2,3-Butanediol [80]	*S. cerevisiae*	2.29 g/L	Pyruvate
Erythritol [25]	*Y. lipolytica*	80 g/L	Glucose-6-phosphate
Mannitol [25]	*Y. lipolytica*	27.6 g/L	Glucose-6-phosphate
Riboflavin [27•]	*Ashbya gossypii*	13.7 g/L	Glucose-6-phosphate
Citric acid [12]	*Y. lipolytica*	154 g/L	Acetyl-CoA
L-Lactic acid [30]	*Candida boidinii*	85.9 g/L	Pyruvate
Ricinoleic acid [34]	*S. pombe*	137.4 μg/mL	Acetyl-CoA
Biodiesel [42•]	*S. cerevisiae*	8.2 mg/L	Acetyl-CoA; Pyruvate
Tetraacetyl phytosphingosine [44]	*Scheffersomyces ciferrii*	2 g/L	Acetyl-CoA
Artemisinin [50••]	*S. cerevisiae* (semi-biosynthesis)	40 g/L	Acetyl-CoA
Patchoulol [53]	*S. cerevisiae*	40.9 mg/L	Acetyl-CoA
Miltiradiene [60•]	*S. cerevisiae*	365 mg/L	Acetyl-CoA
Astaxanthin [64]	*Phaffia rhodozyma*	1.6 mg/L	Acetyl-CoA
Resveratrol [70]	*S. cerevisiae*	391 mg/L	Phosphoenolpyruvate
Vanillin [75]	*S. cerevisiae*	500 mg/L	Phosphoenolpyruvate
Muconic acid [76•]	*S. cerevisiae*	140 mg/L	Phosphoenolpyruvate
Aureothin [77•]	*S. cerevisiae*	6.1 mg/L	Acetyl-CoA
Penicillin [84]	*Hansenula polymorpha*	1 mg/L	Glyceraldehyde 3-phosphate; Pyruvate

transportation fuels [14]. Yeasts have a distinct advantage in this arena due to limited byproducts and a high tolerance to ethanol and similar alcohols when compared to *E. coli*. Significant efforts have been made to harness the power of *Saccharomyces* to produce high yields of alcohols (often near theoretical yields of 0.51 g ethanol/g glucose). Efforts to engineer this organism to use lignocellulosic biomass and alternative sugars have been met with recent success [15]. As examples, *S. cerevisiae* was engineered through cell surface minicellulosomes display for simultaneous saccharification and fermentation of crystalline cellulose to ethanol with titer of 1.4 g/L [16]. Additionally, *S. cerevisiae* has also been engineered to coferment mixtures of xylose and cellobiose and the cofermentation leads to an increase of ethanol productivity from 0.27 g/L h to 0.65 g/L h [17••]. Furthermore, efforts have extended beyond *S. cerevisiae* to yeasts like native xylose fermenter *Scheffersomyces stipitis* for ethanol production [18] and even more exotic yeasts such as *Spathaspora passalidarum* [19].

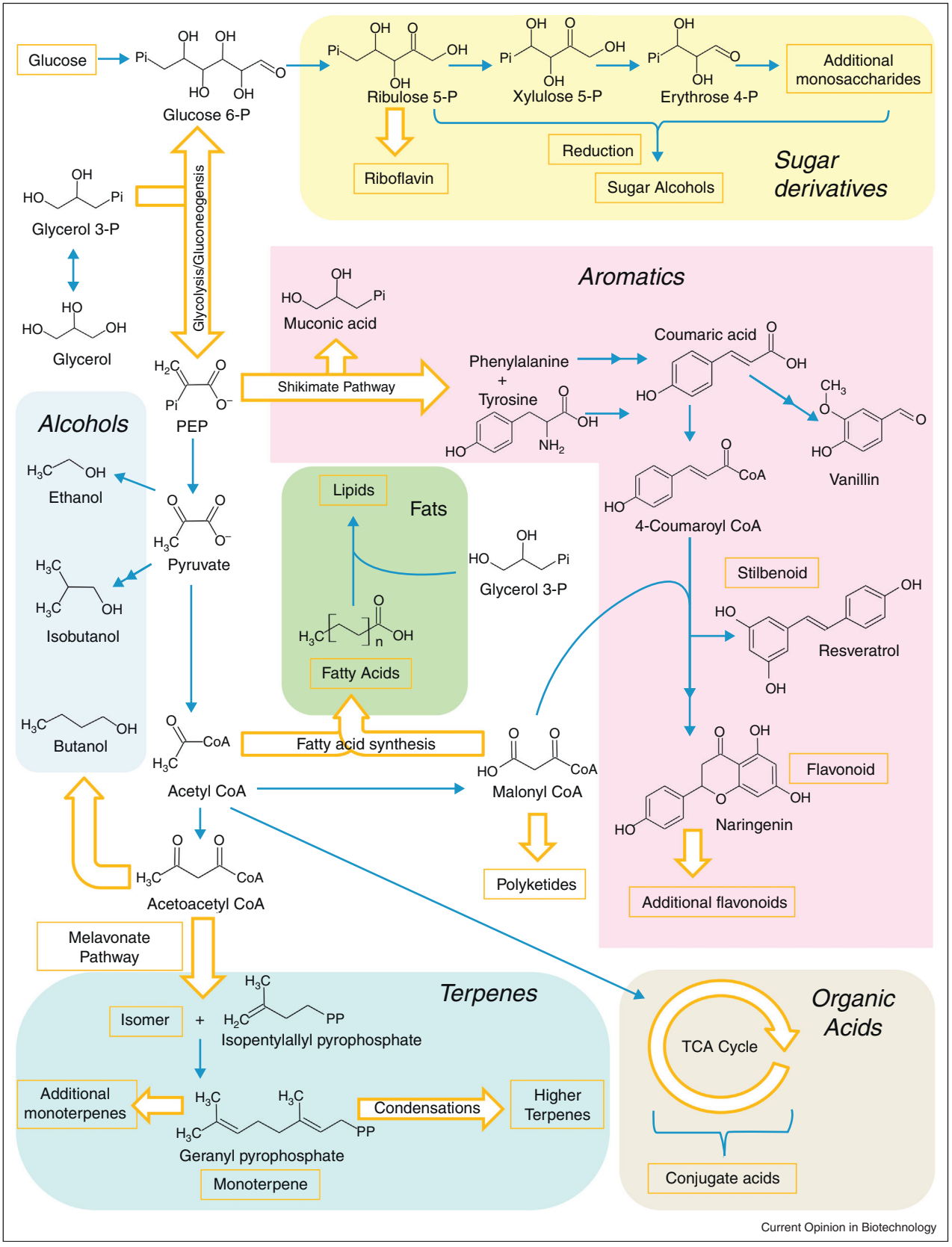
Beyond ethanol, higher alcohols are attractive due to advantages over ethanol such as higher energy density and lower hygroscopicity. The first butanol biosynthetic pathway (from galactose as a carbon source) in *S. cerevisiae* was achieved with a production of 2.5 mg/L [20]. For isobutanol production, the valine pathway was altered through gene overexpressions [21] and eventually led to titers as high as 143 mg/L [22]. The production was further improved (to 0.63 g/L) by relocating the valine biosynthetic enzymes into the cytosol [23•]. A recent report achieved yields of 0.64 g/L isobutanol, 0.1 g/L isopentanol and 0.12 g/L 2-methyl-1-butanol by compartmentalization of the synthesis pathway in yeast mitochondria [11•]. These results highlight that yeast can serve as an interesting platform for alcohol production, but further work is needed to achieve comparable titers of higher-chain alcohols when compared to *E. coli*.

Sugar derivatives

Sugar alcohols and riboflavin are two major compounds synthesized from sugar substrates that are diverted from the pentose phosphate pathway (Figure 1). Sugar alcohols are calorie-free, nonfermentable versions of sugars that can be used as sweetening or flavor additives. Reductase-based pathways ultimately convert fermentable sugars to their respective alcohols. Strain engineering has improved titers as demonstrated by 8.5-fold increases in xylitol and ribitol through expression of *S. stipitis* xylitol dehydrogenase in a transketolase-deficient recombinant *S. cerevisiae* strain [24]. Sugar alcohols have also been produced in the nonconventional yeast *Y. lipolytica*. In this organism, high production of erythritol (80 g/L) and mannitol (28 g/L) was achieved from a glycerol feedstock [25].

Riboflavin, known as vitamin B2, plays a key role in human health and can be derived from the pentose phosphate pathway intermediate ribulose 5-phosphate. Unconventional yeasts *Ashbya gossypii* and *Candida famata* have been used as hosts for riboflavin production with titers of 14 g/L for *Ashbya gossypii* [26,27•]. Collectively, sugar alcohols and riboflavin are chemicals produced with ease by yeast and are great metabolic engineering targets.

Figure 1



Interconnected biosynthetic pathways of value added chemicals.

Organic acids

Organic acids are a central chemical building block in a bioeconomy. Many organic acids produced in yeasts are conjugate acids of citric acid cycle intermediates (Figure 1). A high tolerance to low pH makes yeasts a popular and natural host for producing organic acid such as citric acid, succinic acid, malic acid, and L-lactic acid. High-yield production of citric acid (154 g/L) was achieved in *Y. lipolytica* on glycerol feedstock in repeated-batch bioreactors [12]. A new two-step process for the production of succinic acid from ethanol has achieved high titers (63 g/L) also in *Y. lipolytica* [28]. In glucose-grown batch cultures, an engineered *S. cerevisiae* strain produced malic acid at titers of up to 59 g/L [29]. Production of L-lactic acid from pyruvate by a metabolic engineered *Candida boidinii* strain can reach nearly 86 g/L [30]. In each of these cases, it is clear that yeasts are highly capable of producing many organic acids of interest at bioprocessing-relevant titers.

Fats and lipid-derived molecules

Fatty acids (FA) and lipids are valuable in the nutrition, cosmetics, and pharmaceuticals industry. FAs are built from acetyl-CoA and lipids are then synthesized by condensing a glycerol-3-phosphate backbone with the finished FA (Figure 1). Oleaginous yeasts can naturally accumulate a high lipid content (as high as 70% dry weight) and engineering efforts can convert these lipids into essential fatty acids and other value-added chemicals [31,32].

FA profile in *S. cerevisiae* was altered through engineering targeting TCA cycle in [33]. High content of ricinoleic acid (137 μg/mL) in fission yeast *Schizosaccharomyces pombe* was achieved simply by introducing *Claviceps purpurea* oleate Δ12-hydroxylase gene and this compound accumulated to 52.6% of total FA [34]. Stearodonic acid and dihomo-γ-linolenic acid were also produced in *S. cerevisiae* through heterologous expression of desaturase and elongase enzymes [35,36]. Studies using crude glycerol and glucose for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregular* and *S. cerevisiae* were achieved [37,38]. Accumulation of conjugated linoleic acid and α-linolenic acid can be achieved by expressing linoleic acid isomerase and Δ12/ω3 desaturases respectively in *Y. lipolytica* [39,40]. Other yeasts such as *Lipomyces starkeyi*, *Rhodosporidium toruloides* and *Rhodotorula glutinis* are also being considered [41].

These fatty acids and lipids can ultimately be converted to other desirable molecules. For example, by utilizing wax synthases from *Marinobacter hydrocarbonoclasticus*, *S. cerevisiae* was engineered to produce biodiesel from fatty acids with a titer of 8.2 mg/L [42•]. 3-Hydroxy-γ-decalactone was also overproduced in *Y. lipolytica* by altering the β-oxidation pathway [43]. High-level production of tetraacetyl phytosphingosine (2 g/L) and rare sphingoid bases (890 mg/kg) was also achieved by metabolic engineering of *Pichia ciferrii* [13••,44]. There is great promise in producing fatty acids, lipids, and derived molecules in yeast, in particular for non-*Saccharomyces* hosts.

Terpenes

Terpenes are produced via polymerization of isopentyl pyrophosphate and its isomer (Figure 1) and present an interesting class of compounds with a wide range of uses from dyes, flavors and fragrances, to pharmaceutical, nutraceuticals, and herbal therapies. Yeasts have been viewed as an attractive host for terpene production due to sufficient pools of precursors and tolerance to plant-derived heterologous proteins.

The first monoterpene produced in the pathway is GPP and the alcohol of this terpene, geraniol, has been produced heterologously in yeast with a titer of 5 mg/L [45]. Another monoterpene alcohol, linalool has also been produced in an engineered, high-yielding wine strain (T_{73}-4) to over 140 μg/L [46]. Recently, the isoprene hydrocarbons have been produced in *S. cerevisiae* by utilizing this pathway [47]. Higher titers have been achieved in yeast with sesquiterpenes, formed by the condensation of two GPPs. Ro *et al.* were the first to synthesize artemisinic acid in yeast and were able to achieve titers of 100 mg/L through pathway engineering and removing ergosterol repression [48]. Additional engineering has further increased yields [49]. In a recent report, amorpha-4,11-diene, a precursor in the artemisinic acid pathway, was produced at high yields in a reengineered *S. cerevisiae* with titers exceeding 40 g/L [50••]. 125 mg/L caryophyllene production was achieved through genetic perturbation [51]. Three other different plant sesquiterpenes (valencene, cubebol, and patchoulol) were synthesized in yeast through pathway engineering efforts [52] and further engineered by taking advantage of the spatial organization of metabolic enzymes to improve patchoulol production up to 41 mg/L [53]. Similar titers have been seen for α-santalene in engineered *S. cerevisiae* [54,55]. Recently, 5.2 g/L of bisabolene has been produced from engineered *S. cerevisiae* using a surrogate screening approach that identified high carotenoid production from the yeast deletion collection [56••].

Higher terpenes (such as β-amyrin, taxadiene, miltiradiene and carotenoids) have nutritional and pharmaceutical value. β-Amyrin, a triperpene was produced at levels of 6 mg/L by expressing β-amyrin synthase from *Artemisia annua* and manipulating key enzymes in the pathway [57]. Triterpene hyper-producers have been created by incorporating detected SNPs in metabolic enzymes to enhance carbon flux [58]. Another notable triterpene is taxadiene, a precursor for taxol, a cancer chemotherapy drug, that was produced in yeast with a titer of 8.7 mg/L [59]. Miltiradiene, a precursor to tanshinones, which are

the bioactive components of a well-known traditional Chinese medicine widely used for treatment of many cardiovascular diseases, has also been produced in yeasts with a modular pathway engineering strategy with 365 mg/L being produced in bioreactors [60•]. Finally, carotenoids have been successfully produced in yeast through the introduction of plant enzymes. Recombinant *S. cerevisiae* has been used for β-carotene production to 5.9 mg/g [61]. *P. pastoris* has also been genetically manipulated to produce β-carotene, thus providing an alternative source for large-scale biosynthesis of carotenoids [62]. Astaxanthin has also been produced in *S. cerevisiae* at a level of 29 μg/g [63], while *Phaffia rhodozyma* has been identified as the best biological source of production with a mutant stain achieving almost 2.6 mg/g production [64]. Thus, terpenes are an interesting class of molecules that yeasts can produce with varying success in titers at the moment.

Aromatics

Aromatics are a versatile class of molecules that have both nutritional and therapeutic values as well as uses as polymer precursors. These molecules are derived from the shikimate pathway which converts phosphoenolpyruvate from the glycolysis pathway and erythrose-5-phosphate from the pentose phosphate pathway into tyrosine and phenylalanine (Figure 1). In contrast to *E. coli*, this pathway has significant, tight regulatory control and cannot be immediately de-bottlenecked by simply removing feedback inhibition. However, since yeast still possesses a higher tolerance toward plant heterologous proteins (esp. cytochrome P450s) and di-acids tolerance, the optimization of yeast for aromatics production continues to be a major, but challenging focus.

Resveratrol is a highly regarded stilbenoid molecule with potent antioxidant abilities and potential life extending properties. Through the expression of plant enzymes 4-coumaroyl-coenzyme A ligase (4CL) and 4-coumaroyl-coenzyme A ligase (STS), resveratrol can be produced from coumaric acid in yeast [65,66]. Production can be increased 15-fold to 5.3 μg/mL by creating 4CL and STS enzyme fusions as well as through a synthetic scaffold approach [66,67]. Additional pathway modifications and codon optimization can increase yields further [68,69]. Finally, by using rich media and an industrial Brazilian sugar cane-fermenting yeast, resveratrol production can be boosted to 391 mg/L [70].

Naringenin, a flavonoid itself and a precursor to additional flavonoids, has been produced to a titer of 28 mg/L in *S. cerevisiae* through the heterologous expression of phenylalanine ammonia lyase (PAL), 4CL, chalcone synthase (CHS) and chalcone isomerase. This yield is 62 times higher than the most efficient *E. coli* strain [71]. Other flavonoids such as genistein, kaempferol, quercetin, chrysin, apigenin, and luteolin were also successfully produced in yeasts when using either phenylalanine or phenypropanoid acid precursors as feedstock [72,73].

De novo biosynthesis of vanillin in *S. pombe* and *S. cerevisiae* has been established through heterologous enzymes complementation and metabolic engineering efforts [74]. Further improvement of vanillin production in *S. cerevisiae* was accomplished through *in silico* guided design and fermenter optimization to achieve a level of 500 mg/L [75]. A recent report on muconic acid production with engineered *S. cerevisiae* achieved titers of over 140 mg/L production, the highest titer of an aromatic derived molecule in a yeast shake-flask condition [76•]. While aromatics and derived molecules are of interest, significant metabolic and regulatory obstacles in yeast must be addressed to compete with bacterial hosts.

Polyketides

Polyketides are formed from malonyl CoA (Figure 1) and have strong therapeutic applications. However, polyketide biosynthesis in eukaryotic cells has just emerged when compared to prokaryotic production. Recently, aureothin and spectinabilin were produced in *S. cerevisiae* via a DNA assembler method [77•]. A rational domain swap experiment using highly reducing polyketide synthases in *Aspergillus oryzae* led to the biosynthesis of an extinct metabolite, bassianin [78]. Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains *Aspergillus terreus* enabled high levels of lovastatin and (+)-geodin to be accumulated [79]. Despite the recent success in producing polyketides in yeast systems, there is promise in using this host system.

Conclusion

Beyond the molecules mentioned above, yeasts have served as hosts for many other molecules including 2,3-butanediol [80], glucosinolates [81], benzylisoquinoline alkaloids [82], cephalosporin [83], penicillin [84], and methyl halide [85]. It is clear that the field is quickly moving beyond simply ethanol fermentations with *Saccharomyces*. While there are some pathways where yeasts are clear and dominant winners when compared with bacteria, there is still more work to be done in the pathway and regulatory engineering arena for many. In particular, titers still lag bacterial counterparts. Yet, the recent advancements in metabolic engineering of *S. cerevisiae* and nonconventional yeast alike mean that employing yeasts as a platform for chemical production is more feasible than ever. Although their native properties make them attractive ideal hosts, clearly more breakthroughs are needed to increase production levels as they remain low for many types of molecules (Table 1). However, with the advances of systems and synthetic biology, potential solutions are within reach [86]. Thus, yeasts are poised to become a major producer of many organic molecules of interest in the near future.

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