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Chapter 4

Advances in Metabolic Engineering of *Saccharomyces cerevisiae* for the Production of Industrially and Clinically Important Chemicals

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Abstract

Sustainable production of chemicals is of increasing importance, due to depletion of petroleum and environmental concerns. In addition to its importance in basic research as a simple, eukaryotic model organism, *Saccharomyces cerevisiae* has long been exploited in industry because of its physiological properties. And today, the development in genetic engineering toolbox and genome-scale metabolic models of *S. cerevisiae* has extended its application range to new products and bioprocesses. In addition, evolutionary engineering strategies have been useful in improving cellular properties of *S. cerevisiae*, such as tolerance to product toxicity and inhibitors. In this chapter, recent metabolic and evolutionary engineering studies that involve *S. cerevisiae* for the production of bulk chemicals and fine chemicals including flavours and pharmaceuticals are reviewed. It was shown that metabolic engineering particularly allowed the improvement of pharmaceuticals production, which will enable economic and large-scale production of many valuable pharmaceuticals. It is clear that *S. cerevisiae* will continue to be an important host for future metabolic engineering and metabolic pathway engineering applications to produce a variety of industrially and clinically important chemicals.

Keywords: pharmaceuticals, adaptive evolution, bulk chemicals, evolutionary engineering, flavours, fine chemicals, glutathione, metabolic engineering, organic acids, *Saccharomyces cerevisiae*

1. Introduction

Metabolic engineering was defined by Bailey [1] as 'the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of

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recombinant DNA technology'. More than 20 years after this first definition as a new scientific discipline, metabolic engineering has become an increasingly important research field of biotechnology. Today, metabolic engineering requires interdisciplinary work that includes molecular biology, applied microbiology, biochemical reaction engineering, biomedical research with the aid of high-throughput analytical tools in 'omics' research and bioinformatics [2].

There are two major approaches in metabolic engineering, as described by Bailey et al. [3], the rational metabolic engineering and inverse metabolic engineering. In rational metabolic engineering, extensive genetic and biochemical information is required on the metabolism or metabolic pathway of interest to make defined genetic manipulations. The cellular physiological responses are also complex. Thus, trying to re-engineer a cellular machine that is too complex and about which there is limited information is a major limitation in rational metabolic engineering. Difficulties in cloning in industrial strains due to the lack of relevant genetic tools, and GMO-concerns of the public regarding food industry are additional issues [2]. The inverse metabolic engineering approach was designed to avoid the above-mentioned limitations. Here, the desired phenotype is identified first, as a 'bottom-up' approach, and then its genetic and/or environmental basis is determined which is the most challenging step. However, owing to the powerful high-throughput analytical technologies in genomics, transcriptomics, proteomics and metabolomics, this step is becoming easier [2, 4]. Thus, without any need for extensive initial information on biochemistry, genetics and regulation on the organism of interest, the desired phenotype can be obtained. Adaptive evolution or evolutionary engineering, which is based on random mutation and selection by systematic cultivation of an initial microbial culture in the presence of a selective pressure to obtain desirable phenotypes [5], is a common inverse metabolic engineering strategy [2].

Metabolic engineering is a key strategy for harnessing microorganisms' ability to produce chemicals from renewable carbon sources. Microbial processes are attractive since they have significantly lower environmental impacts than the petroleum-based processes. However, the former is primarily an economic challenge. Therefore, it is vital to develop superior strains with improved yield, titer and productivity by engineering microbial physiology, stress response and metabolism [6]. Considering the market value of chemical products based on petroleum, the cost-competitive bio-based products, once achieved, would have significant economic value as replacements. It is estimated that the global market share of bio-based chemicals will rise from 2% in 2008 to 22% in 2025 [7].

In this chapter, we focused on the recent metabolic engineering studies that involve the baker's yeast, *Saccharomyces cerevisiae*, for the production of industrially and clinically important compounds. *S. cerevisiae* has many advantages to be used in metabolic engineering studies: it has 'Generally Recognized as Safe' (GRAS) status, and there is extensive information on its genetics, physiology and biochemistry. Besides being a common industrial microorganism for ethanol fermentation, baking, brewing, etc., *S. cerevisiae* has been regarded as a versatile cell factory for the production of a wide range of natural compounds by manipulation of the endogenous pathways and/or integration of heterologous pathways. In this review, metabolic

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engineering studies with *S. cerevisiae* are divided into two major categories: production of bulk chemicals, and production of fine chemicals including flavours and pharmaceuticals. Regarding the production of bulk chemicals, examples of organic acids that have potentials to be produced by fermentation at large-scale were discussed. As fine chemicals, glutathione and a variety of secondary metabolites used in food, cosmetic and health industries were discussed.

2. Production of bulk chemicals

The oil refinery is currently the major source of bulk chemicals such as solvents and polymer precursors. A significant portion of petroleum is used in the chemical catalysis for the production of chemicals and plastics [8]. However, in recent years, microbial production of chemicals based on renewable sources, such as biomass, has become important as a part of the efforts to reduce demand on diminishing petroleum and to reduce hazardous wastes. In addition, biotechnology makes new chemical monomers accessible, which are otherwise inaccessible due to high production cost [9].

In bio-refineries, the biomass is the first converted into simple sugars and then to valuable chemicals. Microorganisms are the main players of the latter conversion. Therefore, the development of a suitable strain for the particular process is needed. As a model yeast, *S. cerevisiae* has been a focus of metabolic engineering studies for the bio-based production of chemicals. 1,4-Diacids (succinic, fumaric and malic), itaconic acid, 3-hydroxypropionic acid and lactic acid are organic acids listed among the high-potential targets for industrial biotechnology [10]. Representative examples for the production of these bulk chemicals by metabolically engineered *S. cerevisiae* are summarized in **Table 1**.

Succinic acid is used in a wide range of industries from food to agriculture. Also, it has been considered as a generic intermediate for the bio-based polymers and can be a substitute of petroleum-derived maleic anhydride, which has a huge market [11]. Therefore, an increasing demand of succinic acid is expected in the future. Currently, it is mainly produced by chemical syntheses, which are based on petrochemical precursors. Biotechnological routes are pursued to achieve a sustainable production of succinic acid. *Anaerobiospirillum succiniciproducens* and *Actinobacillus succinogenes* are natural succinic acid producers. However, these organisms are prokaryotes that favour neutral pH for growth and require neutralization and a cost-additive product recovery process. In addition, there is a lack of suitable genetic tools for these organisms [12]. Although *S. cerevisiae* is not a natural producer of succinic acid as an end product, there have been efforts to metabolically engineer *S. cerevisiae,* since it has favourable properties such as the ability to operate at low pH values [13]. In general, the tricarboxylic acid (TCA) cycle and glyoxylate shunt are the focus of these studies. In order to redirect oxidative TCA pathway, elimination of succinate and isocitrate dehydrogenases has been proposed as a strategy. A yeast strain with disturbed TCA cycle due to deletions of *SDH1, SDH2, IDH1, IDP1*, produced succinic acid at a yield of 0.11 mol/mol glucose in shake

Bulk chemical produced	Representative studies and their strain improvement strategy [reference no]
Succinic acid	Disturbance of the citric acid cycle by deleting *SDH1, SDH2, IDH1, IDP1* [14] Disabled serine synthesis from glycolysis through a triple deletion of *SDH1*, *SER3* and *SER33* [15] Enhanced succinic acid export via heterologous expression of *MAE1* from *Schizosaccharomyces pombe* in *Saccharomyces cerevisiae SDH1*- and *SDH2*-disrupted strains [16]
Itaconic acid	Overexpression of *CAD* with a synthetic hybrid promoter and enhancement of flux towards the citric acid cycle by the sequential deletion of the *ADE3*, *BNA2* and *TES1* genes [19]
3-Hydroxypropionic acid	Reconstruction of malonyl-CoA to 3-HP pathway via expression of *MCR* from *Sulfolobus tokodaii* and *HPDH* from *Metallosphaera sedula* and increased precursor and cofactor availability [23] Reconstruction of β-alanine to 3-HP pathway via coexpression of *BAPAT* from *Bacillus cereus* and *HPDH* from *Escherichia coli* and redirection of flux towards β-alanine by overexpressing *PAND* from *Tribolium castaneum* [24] Reconstruction of malonyl-CoA to 3-HP pathway via coexpression of *MCR* from *Chloroflexus aurantiacus* and an inhibition-deficient *ACC1* and optimization of acetyl-CoA supply by overexpressing native *PDC1, ALD6, and ACS* from *Salmonella enterica* [25] Adaptive laboratory evolution for improved tolerance to 3-HP at pH 3.5 [45]
Lactic acid	Expression of genome-integrated *L-LDH* from bovine under *PDC1* promoter and inactivation of *PDC1* [28] Expression of genome-integrated *D-LDH* from *Leuconostoc mesenteroides* subsp. *mesenteroides* under *PDC1* promoter and inactivation of *PDC1* [29] Deletion of *PDC1* and expression of multiple copies of *L-LDH* from bovine [30] Inhibition of L-LDH consumption by deletion of *DLD1* and *JEN1*, elimination of ethanol and glycerol production by deleting *PDC1*, *ADH1*, *GPD1* and *GPD2*, and improvement of lactic acid tolerance by adaptive evolution and overexpression of *HAA1* [31] Overexpression of *HXT1* and *HXT7* hexose transporters [32] Repression of ethanol production by deleting *PDC1* and *ADH1* and enhanced acetyl-CoA supply by the introduction of the genes encoding acetylating acetaldehyde dehydrogenase enzyme from *Escherichia coli* [33] Enhancement of lactic acid transport by expressing *JEN1* and *ADY1* [34] Expression of *ESBP6*, a novel target isolated by screening a multi-copy yeast genomic DNA library [35]

Table 1. Bulk chemical production by metabolically engineered *S. cerevisiae*.

flask cultures [14]. A computational pathway prediction algorithm has been utilized to identify multiple gene deletion targets to redirect carbon fluxes towards succinic acid [15]. Three deletion targets, *SDH3*, *SER3* and *SER33*, were identified to couple succinic acid production to biomass formation. This strategy was based on the elimination of succinic acid consumption by the deletion of *SDH3* encoding cytochrome b subunit of succinate dehydrogenase. The serine biosynthesis was also disrupted by the deletions of *SER3* and *SER33*, which are paralogs encoding 3-phosphoglycerate dehydrogenase. Therefore, serine and glycine production were linked to succinic acid production via glyoxylate pathway. However, the engineered strain required glycine to be supplemented in the medium. Further, two successive laboratory evolution experiments for glycine prototrophy and faster growth were performed with this strain. Finally, overexpression of isocitrate lyase, Icl1p, in the evolved strain, resulted

in a succinic acid yield of 0.07 mol/mol glucose under aerobic conditions without glycine addition. Metabolic profiling analysis of a succinic acid-producing recombinant *S. cerevisiae* hinted a metabolic engineering strategy involving expression of a malic acid transporter from *Schizosaccharomyces pombe* (*MAE1)* to export succinic acid out of cells [16].

Itaconic acid has currently application in the manufacture of pharmaceuticals, adhesives and resins. In addition, its polymerized form (polyitaconic acid) has potentials as a replacement of acrylic acid in the development of superabsorbents [17], and can be used in contact lenses, detergents and cleaners [18]. *Aspergillus terreus* is the present organism of choice for the industrial fermentation of itaconic acid. However, the process bears some constraints due to inherent characteristics of *A. terreus*, such as inhibition in the media and sensitivity to shear stress [19]. Kanamasa et al. isolated *cis*-aconitic acid decarboxylase (CAD), which is the key enzyme in the conversion of *cis*-aconitate to itaconic acid in *A. terreus*, and its heterologous expression in *S. cerevisiae* showed the possibility of itaconic acid production in yeast [20]. Blazeck et al. utilized a synthetic hybrid promoter carrying an enhancer and a core promoter module to optimize CAD expression in *S. cerevisiae* [19, 21]. A genome-wide metabolic model of the yeast was used to identify gene deletion targets to further increase the itaconic acid titer. Three sequential rounds of genome scan *in silico* highlighted three deletion targets; cytoplasmic trifunctional C1-tetrahydrofolate (THF) synthase, a putative tryptophan 2,3-dioxygenase or indoleamine 2,3-dioxygenase and a peroxisomal acyl-CoA thioesterase, encoded by *ADE3, BNA2* and *TES1*, respectively. The deletions rewired metabolic flux towards TCA cycle and enhanced itaconic acid titer (168 mg/L). However, further efforts are necessary to redirect carbon flux towards itaconic acid production in the yeast to approach titers obtained in *Aspergillus* species (>80 g/L).

3-Hydroxypropionic acid (3-HP) is another important platform chemical which can be produced from either sugars or glycerol and can be converted to 1,3-propanediol, acrylic acid, malonic acid, and acrylamide. 3-HP derivatives have a variety of applications in super absorbent polymers, surface coatings, adhesives and paints [11]. Although there are biological pathways to 3-HP via either glycerol, lactate, malonyl-CoA or β-alanine intermediates, no organism is known to produce it as an end product [22]. The pathways based on malonyl-CoA and β-alanine have been constructed in *S. cerevisiae* [23, 24]. Chen et al. evaluated different malonyl-CoA reductases. Malonyl-CoA reductase (MCR_{Ca}) from *Chloroflexus aurantiacus* was expressed in the yeast for the conversion of malonyl-CoA to 3-HP in a two-step reduction reaction. Further, carbon flux was redirected towards 3-HP through increasing the levels of malonyl-CoA and its immediate precursor, acetyl-CoA. For this purpose, native *ADH2* (alcohol dehydrogenase) and *ALD6* (NADP-dependent aldehyde dehydrogenase), and synthetic $acs^{L641P}{}_{SE}$ (acetylation-insensitive acetyl-CoA synthetase from *Salmonella enterica*) were over-expressed to increase the level of acetyl-CoA. The cellular concentration of malonyl-CoA was increased by over-expression of *ACC1* (acetyl-CoA carboxylase), which is the sole enzyme in the conversion of acetyl-CoA to malonyl-CoA. Finally, 3-HP was produced at a titer of 463 mg/L when the production was coupled with enhanced supply of electron donor of MCR_{Ca} (NADPH) by heterologous expression of GAPNp (a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*) [23]. In another study, a significant improvement of 3-HP production was achieved when multiple copies of MCR were integrated

into the yeast genome and a modified ACCp with phosphorylation deficiency was expressed. Finally, engineering of the redox metabolism of this strain produced 3-HP at a titer of 9.8 g/L in a glucose-limited, fed-batch system [25]. Borodina et al. utilized genome-scale modelling to compare the two biosynthetic routes in terms of maximum theoretical yields and identified β-alanine pathway as a more favourable route. They implemented the biosynthesis of 3-HP from glucose via β-alanine through coexpression of β-alanine-pyruvate aminotransferase from *Bacillus cereus* and 3-hydroxypropanoate dehydrogenase from *Escherichia coli*. Further, carbon-flux was redirected towards β-alanine by the supply of L-aspartate, the immediate precursor of β-alanine. The final strain yielded 3-HP at a titer of 13.7 g/L in glucose-limited fed-batch cultivation. In a similar fashion, production of 3-HP via both malonyl-CoA and β-alanine pathway was reported in a xylose-utilizing *S. cerevisiae* [24].

Lactic acid is a well-known fermentation product which is already widely used in food, cosmetics and pharmaceutical industries. Lactic acid derived from biomass is also valued as a monomer in the development of bioplastics [26]. Lactic acid bacteria, especially, *Lactobacillus* species, are often employed in lactic acid production. For large-scale lactic acid production, fermenting microorganisms with high acid tolerance, simple nutritional requirements and capability of growth at high cell density are pursued [27]. To this end, *S. cerevisiae* was engineered for lactic acid production by integrating lactate dehydrogenase (*LDH*) gene into its genome [28, 29]. Reduction in ethanol and glycerol production is desirable to direct metabolite fluxes to lactic acid production. Therefore, deletions of *PDH* encoding pyruvate dehydrogenase, *ADH* encoding alcohol dehydrogenase and *GPD1* encoding glycerol-3-phosphate dehydrogenase were reported to improve lactic acid production in *LDH*-expressing yeast strains [30, 31]. Another approach was the improvement of cell growth either by an increased glucose uptake via overexpression of hexose transporters (*HXT1* and *HXT7*) or an enhanced acetyl-CoA supply through implementing an acetyl-CoA synthesis pathway from *E. coli* in lactic acid-producing *S. cerevisiae* [32, 33]. In addition, elimination of NADH-consuming reactions through deletions of *NDE1* and *NDE2* encoding mitochondrial external NADH dehydrogenases was shown to improve lactic acid production due to increased cofactor availability. The yeast strains that expressed *JEN1* and *ADY2* encoding monocarboxylate permeases constitutively had improved lactic acid production due to higher efflux of lactic acid [34]. Recently, screening of a multi-copy genomic DNA library revealed a novel protein (ESBP6) involved in lactic acid adaptation response, although having a low similarity to monocarboxylate permeases [35]. Lactic acid accumulation under low pH conditions has detrimental effects on yeast cells. Therefore, tolerance to weak acids is another target to achieve high levels of organic acids like lactic acid. A recombinant *LDH*-expressing yeast strain was subjected to adaptive laboratory evolution in the presence of gradually increased D-lactic acid levels. A lactate over-producing strain was obtained with additional copies of *LDH* and *HAA1*, encoding a transcription activator involved in lactic acid stress, and a titer of 112 g/L was achieved in fed-batch cultivation [31].

Product toxicity is a major obstacle for achieving high titers of the target chemicals such as organic acids, aromatic substances and antibiotics [36]. There is limited knowledge about the molecular basis of the product toxicity and tolerance to enable a rational prediction of genetic changes [37]. David et al. developed, for the first time, a hierarchical dynamic pathway control

system involving a two-stage fermentation concept and the use of a metabolic sensor in *S. cerevisiae* [38]. The growth and production phases were decoupled to allow sufficient biomass formation before accumulation of the product beyond toxic levels. In addition, they designed a metabolite sensor based on prokaryotic fapR-fapO system to regulate expression of pathway enzymes in relation to availability of metabolite pools during the production phase. Efficiency of this concept was demonstrated in 3-HP production, which was increased by 10-fold in titers. A more common, alternative approach used against product toxicity or toxic/inhibitory compounds is evolutionary engineering. It is particularly useful for obtaining genetically complex microbial phenotypes such as tolerance to inhibitors/toxic compounds or various stress types [39]. Successful results were obtained by our research group, regarding evolutionary engineering of multi-stress resistant [40], cobalt-resistant [41, 42], nickel-resistant [43], and ethanol-tolerant [44] *S. cerevisiae*. Another example for the use of evolutionary engineering against product toxicity involves adaptive evolution for lactic acid tolerance in *S. cerevisiae* [31]. Similarly, Kildegaard et al. isolated *S. cerevisiae* strains with resistance to 3-HP through laboratory evolution. Genome sequencing of the evolved strains and subsequent functional analyses identified a relevant mutation in *SFA1* gene (*S*-(hydroxymethyl) glutathione dehydrogenase) related to 3-HP tolerance [45].

3. Production of fine chemicals

Plant secondary metabolites hold the potential to be used as pharmaceuticals, cosmetic and food ingredients. However, the yield of these molecules when extracted from natural producers is not in sufficient amounts to meet industrial demands. In addition, chemical synthesis of these complex structures often requires multiple reaction steps and is not a commercially attractive route due to low product yields [46]. Currently, advances in metabolic engineering allowed commercial-scale microbial production of a number of fine chemicals [47–49]. Besides, there is an ongoing academic interest for reconstitution of biosynthetic pathways of several natural products, including complex pathways, in *S. cerevisiae.* Discovery of gene clusters involved in the biosynthesis of secondary metabolites have enhanced progress in microbial production of these molecules [50]. Computational studies have also been conducted to optimize heterologous production in a variety of industrial host microorganisms including *S. cerevisiae,* which involved application of flux balance analysis on genome-scale models for different hosts to identify the optimum host for production [51].

3.1. Flavours

Compounds belonging to isoprenoid and phenolics type of secondary metabolites are valued as natural fragrances and flavours. Flavour compounds can be produced from sugars (*de novo* synthesis) or from specific precursors (bioconversion) by using microorganisms.

Vanillin, a phenolic aldehyde, is one of the first flavour compounds produced in microbial hosts at commercial-scale. Current state of the microbial production of vanillin based on various precursors and the available production hosts have been recently reviewed by Gallage

and Møller [52]. *De novo* biosynthesis of vanillin from glucose in *S. cerevisiae* has also been reported [53]. A multi-step conversion of a shikimate pathway intermediate (3-dehydroshikimate) to vanillin has been achieved through heterologous expression of four genes from *Podospora pauciseta, Nocardia iowensis, Corynebacterium glutamicum* and *Homo sapiens*. Once the vanillin biosynthesis was established, genome-scale metabolic modelling was used to identify gene deletion targets to improve vanillin production in *S. cerevisiae*. *PDC1* and *GDH1* deletions resulted in a five-fold increase in production (500 mg/L) [47].

p-Coumaric acid, a hydroxyl derivative of cinnamic acid, is a commercially attractive end-product and a platform compound for flavonoids, polyphenols and polyketides, as well. Rodriguez et al. achieved high titers (2 g/L) of *p*-coumaric acid as the end-product in *S. cerevisiae*, through optimization of native aromatic amino acid biosynthesis [54]. The competing pathways were eliminated while enhancing production pathways by the expression of feedback resistant enzymes in combination with gene deletions and overexpression of analogue enzymes from *E. coli*.

β-Ionone is an apocarotenoid that is naturally present in raspberries. In *S. cerevisiae*, *de novo* synthesis of β-ionone was reported [55]. Beekwilder et al. constructed a β-carotene synthesis pathway via farnesyl diphosphate (FPP) intermediate through polycistronic expression of genes from *Xanthophyllomyces dendrorhous*. The pathway was further extended, for the first time, to produce β-ionone by the expression of a carotenoid-cleavage dioxygenase (*CCD1*) from raspberry.

2-Phenyl ethanol (2-PE) is another economically attractive flavour compound with a rose-like scent. Ehrlich pathway is involved in the bioconversion of phenylalanine to 2-phenyl ethanol within *S. cerevisiae*. Elimination of allosteric feedback regulation on the aromatic amino acid biosynthesis resulted in an increase of up to 200-fold in the production of aromatic compounds, including 2-PE. Romagnoli et al. constructed a deletion library of non-essential genes in *S. cerevisiae* by Synthetic Genetic Array (SGA) technology and identified that *ARO8* encoding an aromatic amino acid transaminase is a target to improve phenylethanol production from glucose [56]. Recently, Shen et al. identified *AAT2* encoding a cytosolic aspartate aminotransferase as another deletion target [57]. Deletion of these two genes in combination with the overexpression of Ehrlich pathway enzymes resulted in a significant improvement in 2-PE production from glucose, at a titer of 96 mg/L.

3.2. Pharmaceuticals

Another major area of metabolic engineering research is the production of clinically important compounds. In this section, examples will be given for the production of a variety of such compounds by metabolically engineered yeast. Representative examples for the production of pharmaceuticals by metabolically engineered *S. cerevisiae* are summarized in **Table 2**.

Glutathione, a naturally occurring tripeptide, is an important compound used in health and cosmetic industries. It is produced by using *S. cerevisiae* at commercial-scale. There has been a remarkable progress in glutathione production by metabolic engineering studies over the last few decades. Improved levels of glutathione production were achieved by *YAP1*

Pharmaceutical produced	Representative studies and their strain improvement strategy [reference no]
Glutathione	Overexpression of *YAP1* [58] Manipulation of the sulphate assimilation pathway by overexpressing *MET14* and *MET16* [59] Improved oxidized glutathione production by overexpression of *GSH1*, *GSH2*, and *ERV1* and the deletion of *GLR1* [60] Adaptive laboratory evolution in the presence of increasing levels of acrolein and screening for enhanced glutathione production [61] Whole-genome engineering via genome shuffling and screening for enhanced glutathione production [62]
Artemisinin/artemisinic acid	Reconstruction of the complete biosynthetic pathway of artemisinic acid, including the three-step oxidation of amorphadiene to artemisinic acid by expression of *CYP71AV1*, *CPR1*, *CYB5*, *ADH1* and *ALDH1* from *Artemisia annua* [48]
Taxol/taxadiene	Expression of a truncated version of the endogenous *tHMG1* and *GGPPS* from *Taxus chinensis* or *Sulfolobus acidocaldarius* together with *TDC1* from *T. chinensis* [66] Prediction of the efficiency of different GGPPS enzymes via computer aided protein modelling [67]
Forskolin	Expression of a promiscuous cytochrome P450 from *Salvia pomifera* [68]
Polyketides	Heterologous expression of 6-MSA synthase gene from *Penicillium patulum* together with PPTases from either *Bacillus subtilis* or *Aspergillus nidulans* [69] Construction of polyketide precursor pathways by expressing *prpE* from *Salmonella typhimurium* and PCC pathway from *Streptomyces coelicolor* [70] Enhanced cofactor supply by expressing *2-PS* from *Gerbera hybrida* [71]
Resveratrol	Reconstruction of a *de novo* pathway by expressing *TAL* from *Herpetosiphon aurantiacus*, *4-CL1* from *Arabidopsis thaliana* and *VST1* from *Vitis vinifera* [49] Expression of *4CL1* from *A. thaliana* and *STS* from *Arachis hypogaea* [73] Expression of *PAL* from *Rhodosporidium toruloides*, *C4H* and *4-CL1* from *A. thaliana*, and *STS* from *A. hypogaea* [74] Expression of 4-coumaroyl-coenzyme A ligase (*4CL1*) from *A. thaliana* and stilbene synthase (*STS*) from *V. vinifera* [75] Overexpression of the resveratrol biosynthesis pathway, enhancement of P450 activity, increasing the precursor supply for resveratrol synthesis via phenylalanine pathway [76]
Dihydrochalcones	Expression of the heterologous pathway genes in a *TSC13*-overexpressing *S. cerevisiae* strain [78]
Alkaloids	Expression of 14 monoterpene indole alkaloid pathway genes from *Catharanthus roseus* and enhanced secondary metabolism to produce strictosidine *de novo* [79] Construction of the complete *de novo* biosynthetic pathway to norcoclaurine by expressing a mammalian TyrH enzyme and DODC from *Pseudomonas putida,* along with four genes required for biosynthesis of its electron carrier cosubstrate [80] Expression of AdoMet-dependent methyltransferase enzymes (6-OMT, CNMT and 4′-OMT) from plant and human origin to produce reticuline from norlaudanosoline [81] Reconstruction of berberine biosynthetic pathway from reticuline by expressing seven relevant heterologous genes [82] Reconstruction of a 10-gene biosynthetic pathway from plant to produce sanguinarine from norlaudanosoline [83] Expression of 16 heterologous plant enzymes to produce noscapine from canadine [84] Reconstruction of a seven-gene pathway for the production of codeine and morphine from (*R*)-reticuline [85] Reconstruction of a *de novo* biosynthetic pathway for thebaine by expression of 21 genes from plants, mammals, bacteria and the yeast [86]

Table 2. Production of pharmaceuticals by metabolically engineered *S. cerevisiae*.

overexpression [58], metabolic engineering of the yeast sulphate assimilation pathway and glutathione biosynthetic pathway [59], overexpression of a novel glutathione export ABC protein (Adp1p, Gxa1p) and the engineered thiol redox metabolism [60]. Also, the inverse metabolic engineering approach was used to increase glutathione production in *S. cerevisiae* [61, 62]. In an evolutionary engineering study, acrolein, a toxic α,β-unsaturated aldehyde, was used as a selection agent. Two rounds of adaptive evolution in the presence of increasing levels of acrolein resulted in evolved strains with acrolein tolerance and up-to 3.3-fold higher glutathione accumulation in comparison to the parental strain [61]. Genome shuffling has also been applied to obtain yeast strains with increased glutathione content. Two rounds of recursive protoplast fusion were performed with the improved strains initially obtained from ultraviolet irradiation and chemical mutagenesis. The strain with highest glutathione content showed 9.9-fold transcriptional up-regulation of glutathione synthetase gene (*GSH-I*) [62].

Terpene derivatives are economically viable molecules that are used in the synthesis of drugs such as the antimalarial agent artemisinin, and the anticancer agent taxol [63]. Several terpenoids have been produced in *S. cerevisiae* by reconstitution of the relevant biosynthetic pathways. As part of efforts to establish a solid source of artemisinin, *S. cerevisiae* was metabolically engineered to produce artemisinic acid, which is an artemisinin precursor [48]. As the microbially produced artemisinic acid was converted to artemisinin by synthetic chemistry methods, that study was reported as a good example for combining biological production by metabolic engineering with production by synthetic chemistry [64]. Paddon et al. have, for the first time, designed a *S. cerevisiae* strain with the complete biosynthetic pathway of artemisinic acid, involving overexpression of the mevalonate pathway enzymes, and achieved commercial-scale titers (25 g/L) [48].

The well-known diterpenoid taxol is an anti-cancer agent [63, 65]. As a first step towards taxol production, *S. cerevisiae* was metabolically engineered for taxadiene biosynthesis [66]. For this purpose, heterologous genes encoding enzymes from the early steps of the taxoid biosynthesis pathway, isoprenoid pathway, were introduced, along with a regulatory factor to inhibit competing pathways. The results were promising enough for taxol production in recombinant microorganisms [66]. By using protein modelling and substrate docking, different geranylgeranyl diphosphate synthases were screened and expressed in a recombinant taxadiene-producing yeast. The yeast strains were compared in terms of their metabolism using metabolomics approach to identify an efficient host for taxadiene production [67].

Forskolin is a labdene diterpene with potentials to be used in the treatment of blood pressure, in weight-loss supplements and in the protection against congestive heart failure. Ignea et al. constructed a yeast platform to produce 11β-hydroxy-manoyl oxide, forskolin precursor. Although the forskolin biosynthetic pathway has not been completely discovered yet, a promiscuous cytochrome P450 from *Salvia pomifera* was identified as a replacement to achieve the synthesis of the forskolin precursor. This study can provide a basis for the biosynthesis of various tricyclic (8,13)-epoxy-labdanes [68].

Polyketides are also a major group of natural products with a wide range of applications as antibiotics, immunosuppressors, cholesterol lowering agents and other drugs [69]. *S. cerevisiae* is known as a suitable production host for simple polyketides. An earlier study demonstrated

the production of a simple polyketide, 6-methylsalicylic acid, by heterologous expression of 6-methylsalicylic acid synthase in *S. cerevisiae* [69]. However, the major challenge in the synthesis of complex polyketides was the lack of polyketide precursor pathways in *S. cerevisiae*. To overcome this, a relevant pathway was introduced into *S. cerevisiae* to produce a precursor for complex polyketides, methylmalonyl-coenzyme A (CoA). This engineered yeast strain had the capability of the production of a triketide lactone, when supplemented with propyldiketide thioester [70]. Since polyketides are derived from acetyl-CoA and malonyl-CoA precursors, an increase in the acetyl-CoA and the cofactor (NADPH) in a yeast strain expressing 2-pyrone synthase (2-PS) from *Gerbera hybrida* led to 6.4-fold higher triacetic acid lactone production, compared to the reference strain [71].

The strategy of engineered precursor pools has also been applied in the production of resveratrol. Resveratrol is a polyketide derivative with potent antioxidant properties and it has been recently brought to market as a bio-product [72]. Earlier reports on the production of resveratrol were based on bioconversion of aromatic precursors such as *p*-coumaric acid and tyrosine by engineered *S. cerevisiae* strains [73, 74]. The highest resveratrol titer achieved by using this approach was obtained by an engineered industrial Brazilian *S. cerevisiae* strain, at a titer of 391 mg/L resveratrol on complex medium supplemented with *p*-coumaric acid [75]. Recently, in order to produce resveratrol from cheaper carbon sources, *de novo* biosynthesis of resveratrol via tyrosine intermediate in *S. cerevisiae* has been established by constructing an engineered pathway, involving tyrosine ammonia-lyase from *Herpetosiphon aurantiacus*, 4-coumaryl-CoA ligase from *Arabidopsis thaliana* and resveratrol synthase from *Vitis vinifera* [49]. To direct flux towards tyrosine, feedback-insensitive *ARO4* encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and *ARO7* encoding a chorismate mutase were overexpressed. To increase the precursor malonyl-CoA, an inactivation-sensitive acetyl-CoA carboxylase was overexpressed. Resveratrol production was further improved by integration of multiple copies of pathway genes, and finally, a titer of 415.65 and 531.41 mg/L resveratrol was obtained in a fed-batch cultivation with glucose or ethanol as the carbon source, respectively [76]. Koopman et al. also focused on deregulation of feedback mechanism of aromatic amino acid biosynthesis for *de novo* production of naringenin, which is an important platform molecule for the production of flavonoids [77].

Dihydrochalcones (DHCs) such as nothofagin, phlorizin and naringin dihydrochalcone are another group of polyketide derivatives with commercial value as antioxidants, antidiabetics or sweeteners. Recently, *de novo* synthesis of DHCs via phloretin intermediate has been reported in *S. cerevisiae* [78]. First, phloretin biosynthesis was achieved with the aid of a side activity of an endogenous double-bond reductase, in combination with heterologous pathway enzymes. To eliminate by-product formation, a chalcone synthase with high substrate specificity was expressed from *Hordeum vulgare*. Commencing with phloretin, several DHC derivatives with antioxidant, antidiabetic and sweetener properties have been obtained through an extension pathway involving methylation or glycosylation by previously known enzymes.

Recently, there have also been many reports on the reconstitution of biosynthetic pathways of alkaloids in yeast. Alkaloids are nitrogen-containing complex molecules with potent biological activity. Currently, there are around 50 alkaloid-based drugs, including the anticancer

drug vincristine, the antitussive agent noscapine and the analgesic codeine. Strictosidine was the first reported plant-derived alkaloid produced *de nov*o in *S. cerevisiae* [79]. Strictosidine is a common intermediate of a list of alkaloids derived from tryptophan in plants, including the antimalarial quinine and anticancer agent vincristine [79]. Brown et al. reconstituted its biosynthetic pathway in *S. cerevisiae*. To enable strictosidine production in yeast, 14 genes from *Catharanthus roseus* were expressed [79]. The flux through the pathway was further improved by integration of additional copies of the relevant endogenous genes and three gene deletions that eliminated competing pathways. *S. cerevisiae* has also been engineered for the production of (*S*)-reticuline, which is a key branch point intermediate in the biosynthesis of a variety of alkaloids, including well-known opioids such as morphine and thebaine [80]. Bioconversion of a commercial substrate norlaudanosoline to reticuline was reported in an engineered yeast strain expressing three different AdoMet-dependent methyltransferase enzymes (6-OMT, CNMT and 4'-OMT) from plant and human origin [81]. Trenchard et al. constructed a route to reticuline which enabled *de novo* synthesis of this molecule via norcoclaurine intermediate, the actual intermediate in plants. The pathway comprised of a modified yeast amino acid biosynthesis pathway, in combination with a heterologous pathway involving seven relevant enzymes [80]. In other studies, *S. cerevisiae* strains were engineered to produce berberine, dihydrosanguinarine and noscapine from norlaudanosoline via reticuline intermediate, through a 7-, 10- and 14-step pathway involving heterologous expression of plant enzymes, respectively [82–84]. Also, the production of codeine and morphine from (*R*)-reticuline was reported by reconstitution of a seven-gene pathway in *S. cerevisiae* [85]. These studies provided a basis towards designing yeast cell factories for *de novo* production of reticuline-derived molecules. Recently, a complete pathway of biosynthesis of opioid thebaine from sugar has been established in *S. cerevisiae* [86]. This work involved a combination of enzyme discovery, protein engineering of a key cytochrome P450 and pathway optimization. The thebaine-producing yeast strains required expression of 21 heterologous genes from plants, mammals, bacteria and yeast. The pathway was also extended through expression of two additional genes from bacteria and plant to produce hydrocodone, a widely prescribed opioid drug.

4. Summary and outlook

For fine chemicals such as amino acids, vitamins, flavours, nutraceuticals, organic acids and fragrances, profit margins are usually not high and could be affected by substrate availability and cost. However, metabolic engineering enabled improvements in production of both pharmaceuticals and fine chemicals which will allow economic and large-scale production of many valuable compounds in near future.

It is obvious that *S. cerevisiae* will continue to be an important host for future metabolic engineering applications. There will be more comprehensive future studies on the production of chemicals by metabolic engineering of *S. cerevisiae.* These metabolic engineering strategies will most likely involve combinations of rational and inverse metabolic engineering approaches by adaptive evolution of recombinant *S. cerevisiae* with engineered metabolic pathways for various substrate utilization. Additionally, more studies on adaptive

evolution and molecular characterization of tolerance to toxic end-products are expected in the future. Similarly, metabolic pathway engineering of *S. cerevisiae* will allow efficient production of more clinically important compounds and fine chemicals. It can be predicted that the advances in systems biology and bioinformatics will make a significant contribution to yeast metabolic engineering.

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