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Chapter

De Novo Synthesis of Plant Natural Products in Yeast

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Abstract

Plant natural products possess versatile biological activities including antiviral, anticancer and hepatoprotective activities, which are widely used in pharmaceutical and many other health-related fields. However, current production of such compounds relies on plant culture and extraction, which brings about severe concerns for environmental, ecological and amount of agricultural lands used. With the increasing awareness of environmental sustainability and shortage of lands, yeasts are engineered to produce natural products, for its inherent advantages such as the robustness, safety and sufficient supply of precursors. This chapter focused on the recent progress of yeast as a platform for the biosynthesis of plant natural products.

Keywords: natural products, flavonoids, alkaloids, terpenoids, terpenoids saponins, biomanufacturing, heterogeneous synthesis, yeast

1. Introduction

Plant natural products were a kind of active compounds including flavonoids, alkaloids, terpenoids and saponins etc. As the main composition of plants secondary metabolites, these compounds play an important role in plant communication and defensing, so these compounds have been widely used as herbicide and pesticide in the agricultural industry [1]. For example, oleanane saponins isolated from *Bellis sylvestris* exhibit strong phytotoxic activity against Aegilops geniculate, and saponins from alfalfa can cause a decrease of food metabolized by *Tenebrio molitor* [2]. These compounds were also the major bioactive constituents of some traditional herbal medicines, such as ginsenosides from ginseng and glycyrrhizin from licorice. In addition, some natural products possess special flavors. For instance, camphor alcohol has distinctive aroma, while glycyrrhetinic acid monoglucuronide and mogroside V have a strong sweetness which are approximately 941-fold and 300-fold of sucrose, respectively [3]. These sweet tasting compounds have been widely used in the food industries for weight loss. As a kind of amphiphilic compounds, saponins can also act as robust foaming agent in aqueous solutions. Based on this trait potential, they have been explored in the cosmetics and detergent industries.

Currently, the production of natural products are mainly based on extraction from plants, which is a low yield, time consuming, labor intensive and environment unfriendly supply way [4]. The inefficient approach could not match the huge demands of market, and further limit the application of these compounds in the pharmaceutical, agricultural, food, cosmetic and detergent industries. Thus, developing novel approaches are of great significance to replace the traditional method.

Producing natural products via microorganism cell factories turned out to be a promising solution. Compared with plants, microbes exhibit many advantages, including fast growing, land saving and controllable. Yeast especially *Saccharomyces cerevisiae* (*S. cerevisiae*) has become a widely used host for producing these products because of its similar intracellular structure with plant cells, such as the inherent endomembrane system for microsomal enzymes to stand on. Moreover, the generally recognized as safe (GRAS) yeast as a robust industrial strain is widely used in food and alcohol production and has a clear genetic background. Typically, nearly 25 g/L artemisinic acid has been produced in *S. cerevisiae*, which indicated that producing natural products by yeast is a potential approach to substitute the traditional supply way.

In this chapter, we systematically illustrate the decoded biosynthetic pathway of flavonoids, alkaloids, terpenoids and terpenoids saponins assisted by yeast. Then briefly summarize the progress of yeast to produce plant natural products. Furthermore, novel strategies and tools used to boost their production were discussed.

2. Synthetic pathways of natural products

Flavonoids and alkaloids are usually derived from the shikimate pathway, which exists in prokaryotic, eukaryotic, and archaeal microorganisms. The synthesis pathway start from the stereo-specific condensation catalyzed by 3-deoxy-Darabino-heptulosonate-7-phosphate synthase to generate 3-deoxy-Darabino-heptulosonate-7-phosphate (DAHP), which is further catalyzed to form chorismate a common precursor for various aromatic compounds, including aromatic amino acids, then these aromatic compound will be converted to flavonoids and alkaloids **Figure 1(a)**.

Terpenes and saponins are usually synthesized from the common five-carbon building blocks, 3-isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized through mevalonic acid (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [5]. These five-carbon units are condensed to geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP). These precursors are then diverted to specialized terpenes by terpene synthases. GPP is converted to monoterpene by

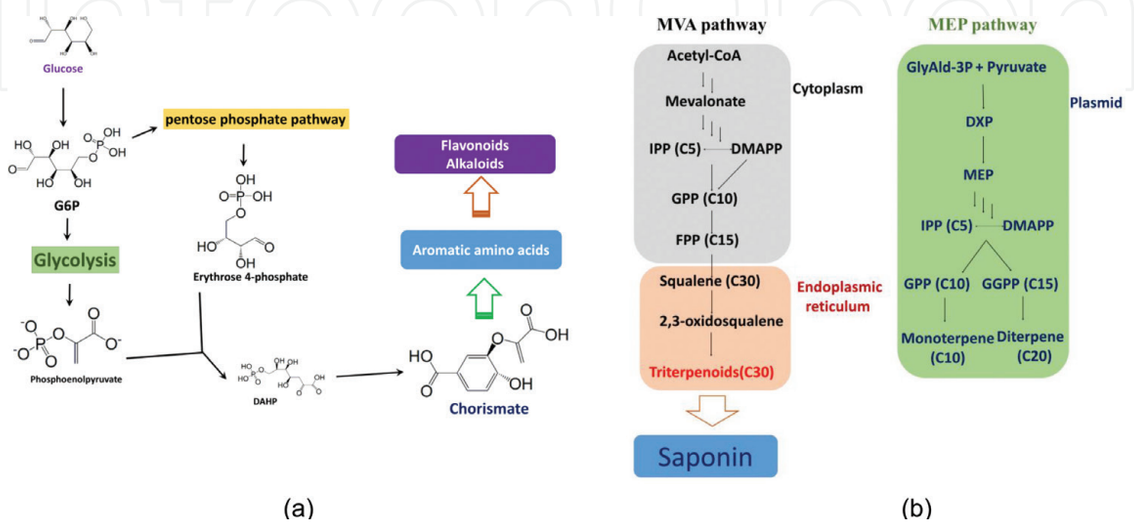

Figure 1.
(a) Scheme for flavonoid and alkaloid biosynthetic pathways. (b) Scheme for terpene and saponins biosynthetic pathways.

monoterpene synthases, GGPP were used to synthesize diterpenes which can be further converted to tretaterpenes, and FPP is the precursor for triterpenes which can be converted to saponins by UGTs **Figure 1(b)**.

In order to produce flavonoids, alkaloids, monoterpene, diterpene and tretaterpene heterogeneously, *E. coli* is widely used for enzyme identification, because of its ease genetic manipulations, whereas the yeast platform was mostly used in triterpene synthesis for its endogenous 2,3-oxidosqualene supplement and inner membrane structure for membrane located plant CYP450 to stand on, which is intensively used and studied in natural products synthesis.

During triterpenoids synthesis, FPP is condensed to 2,3-oxidosqualene, which is subsequently cyclized to polycyclic triterpenoid skeletons by oxidosqualene cyclases (OSCs). These molecules are oxidized by CYP450s forming aglycones, which are further glycosylated to triterpenoid saponins by UGTs.

Cyclization of 2,3-oxidosqualene by OSCs, was the first step in triterpenoid biosynthesis. Because of the efficient 2,3-oxidosqualene supplement, most OSCs was verified by directly expressing in yeast [6]. Plants OSCs always possess promiscuous activities, and could cyclize 2,3-oxidosqualene to different conformations simultaneously. For example, amyrin synthase from *Glycyrrhiza uralensis* can produce not only α-amyrin but also β-amyrin [7]. Due to the multiple-activity of OSCs, more than 100 kinds of triterpenoid skeletons could be generated during cyclization. This was considered as a part of plant defense, for many defensive substances could be produced by one process, which can alleviate the metabolic burden of the plants. However, the promiscuity of OSCs would lead to undesired structural analogue [8].

The triterpenoid skeletons could be further oxidized by CYP450s, introducing active groups such as hydroxyl, carboxyl or epoxy groups [9]. The CYP450s decoding process was complicated, for the identification of CYP450s need much chemical and bioinformatics information, and the membrane located plant CYP450s is hard to express in *E. coli*. Hence, *S. cerevisiae* together with chromatography mass spectrum and NMR play an important role in the decoding of plant biosynthetic pathways involving CYP450s especially for the biosynthesis of triterpenoids and their saponins. To date, 48 CYP450s have been identified involved in triterpenoid saponins biosynthesis. They were summarized in **Table 1**.

No.	Name	Accession number	Plant species	Substrate	Loci
1	CYP51H10	ABG88965.1	*Avena strigosa*	β-amyrin	C-12, 13, 16β
2	CYP71D353	AHB62239.1	*Lotus japonicus*	lupeol	C-20
3	CYP72A61v2	BAL45199.1	*Medicago truncatula*	24-OH-β-amyrin	C-23
4	CYP72A63	BAL45200.1	*Medicago truncatula*	β-amyrin	C-30
5	CYP72A67	ABC59075.1	*Medicago truncatula*	oleanolic acid	C-2
6	CYP72A68v2	BAL45204.1	*Medicago truncatula*	oleanolic acid	C-25
7	CYP72A69	BAW35014.1	*Glycine max*	β-amyrin	C-21
8	CYP72A154	BAL45207.1	*Glycyrrhiza uralensis*	β-amyrin	C-30
9	CYP87D16	AHF22090.1	*Maesa lanceolata*	β-amyrin	C-16α
10	CYP88D6	AQQ13664.1	*Glycyrrhiza uralensis*	β-amyrin	C-11
11	CYP93E1	BAE94181.1	*Glycine max*	β-amyrin	C-24
12	CYP93E2	ABC59085.1	*Medicago truncatula*	β-amyrin	C-24

No.	Name	Accession number	Plant species	Substrate	Loci
13	CYP93E3	BAG68930.1	*Glycyrrhiza uralensis*	β-amyrin	C-24
14	CYP93E4	AIN25416.1	*Arachis hypogaea*	β-amyrin	C-24
15	CYP93E5	AIN25417.1	*Cicer arietinum*	β-amyrin	C-24
16	CYP93E6	AIN25418.1	*Glycyrrhiza glabra*	β-amyrin	C-24
17	CYP93E7	AIN25419.1	*Lens culinaris*	β-amyrin	C-24
18	CYP93E8	AIN25420.1	*Pisum sativum*	β-amyrin	C-24
19	CYP93E9	AIN25421.1	*Phaseolus vulgaris*	β-amyrin	C-24
20	CYP716A12	ABC59076.1	*Medicago truncatula*	α-amyrin, β-amyrin, lupeol	C-28
21	CYP716A14v2	AHF22083.1	*Artemisia annua*	α-amyrin, β-amyrin	C-3
22	CYP716A15	BAJ84106.1	*Vitis vinifera*	α-amyrin, β-amyrin, lupeol	C-28
23	CYP716A17	BAJ84107.1	*Vitis vinifera*	α-amyrin, β-amyrin, lupeol	C-28
24	CYP716A44	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-28
25	CYP716A46	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-28
26	CYP716A47	AEY75213.1	*Panax ginseng*	dammarenediol-II	C-12
27	CYP716A52v2	AFO63032.1	*Panax ginseng*	β-amyrin	C-28
28	CYP716A53v2	AFO63031.1	*Panax ginseng*	dammarenediol-II	C-20
29	CYP716A75	AHF22088.1	*Maesa lanceolata*	β-amyrin	C-28
30	CYP716A78	ANY30853.1	*Chenopodium quinoa*	α-amyrin, β-amyrin, lupeol	C-28
31	CYP716A79	ANY30854.1	*Chenopodium quinoa*	α-amyrin, β-amyrin, lupeol	C-28
32	CYP716A80	ALR73782.1	*Barbarea vulgaris*	α-amyrin, β-amyrin, lupeol	C-28
33	CYP716A81	ALR73781.1	*Barbarea vulgaris*	α-amyrin, β-amyrin, lupeol	C-28
34	CYP716A83	AOG74832.1	*Centella asiatica*	β-amyrin	C-28
35	CYP716A86	AOG74831.1	*Centella asiatica*	β-amyrin	C-28
36	CYP716A140	AOG74836.1	*Platycodon grandiflorus*	β-amyrin, 24-OH-β-amyrin	C-28
37	CYP716A141	AOG74838.1	*Platycodon grandiflorus*	β-amyrin, 24-OH-β-amyrin	C-28
38	CYP716A180	–	*Betula platyphylla*	lupeol	C-28
39	CYP716A244	APZ88353.1	*Eleutherococcus senticosus*	β-amyrin	C-28
40	CYP716A254	–	*Anemone flaccida*	β-amyrin	C-28
41	CYP716AL1	AEX07773.1	*Catharanthus roseus*	α-amyrin, β-amyrin, lupeol	C-28
42	CYP716C11	AOG74835.1	*Centella asiatica*	oleanolic acid	C-2
43	CYP716E41	AOG74834.1	*Centella asiatica*	maslinic acid	C-6

No.	Name	Accession number	Plant species	Substrate	Loci
44	CYP716E22	–	*Solanum lycopersicum*	α-amyrin, β-amyrin	C-6
45	CYP716S5	AOG74839.1	*Platycodon grandiflorus*	β-amyrin, oleanolic acid	C-12, 13
46	CYP716Y1	AHF45909.1	*Bupleurum falcatum*	α-amyrin, β-amyrin	C-16α
47	CYP716A1	AED94045.1	*Arabidopsis thaliana*	β-amyrin	C-28
48	CYP716A2	BAU61505.1	*Arabidopsis thaliana*	α-amyrin	C-22

Table 1.
Overview of plant CYP450s in triterpenoid saponins biosynthesis.

Glycosylation is the last step of triterpenoid saponins biosynthesis that links hydrophilic sugar moieties to the hydrophobic aglycone by UGTs. By glycosylation, various monosaccharide units (including glucose, glucuronic acid, galactose, rhamnose, xylose and arabinose, etc.) could be linked to aglycone at the positions C-3, C-28, C-4, C-16, C-20, C-21, C-22 and/or C-23. The introduced of sugar moieties could improve triterpenoid saponins bioactivities. In view of the tremendous amounts of UGTs in plants, more than 120 genes encoding family 1 UGTs have been identified in *Arabidopsis thaliana* genome [10]. However, Decoding the specific UGT involved in target triterpenoid saponins biosynthesis was very difficult. So far, only 23 UGTs have been identified, which involved in triterpenoid saponins biosynthesis. They were summarized in **Table 2**.

No.	Name	Accession number	Plant species	Substrate	Loci
1	UGT71G1	AAW56092.1	*Medicago truncatula*	Medicagenic acid UDP- glucose	C-3, 28
2	UGT73AD1	ALD84259.1	*Centella asiatica*	Asiatic acid, Madecassic acid UDP- glucose	C-28
3	UGT73AE1	AJT58578.1	*Carthamus tinctorius*	Glycyrrhetinic acid UDP- glucose	C-3
4	UGT73AH1	AUR26623.1	*Centella asiatica*	Asiatic Acid UDP- glucose	C-28
5	UGT73C10	AFN26666.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP- glucose	C-3
6	UGT73C11	AFN26667.1	*Barbarea vulgaris*	Glycyrrhetinic acid, Oleanolic acid UDP- glucose	C-3
7	UGT73C12	AFN26668.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP- glucose	C-3
8	UGT73C13	AFN26669.1	*Barbarea vulgaris*	Hederagenin, Oleanolic acid UDP-glucose	C-3
9	UGT73F2	BAM29362.1	*Glycine max*	Saponin A0-gα UDP-xylose	C-22
10	UGT73F3	ACT34898.1	*Medicago truncatula*	Hederagenin UDP- glucose	C-28
11	UGT73F4	BAM29363.1	*Glycine max*	Saponin A0-gα UDP-xylose	C-22
12	UGT73F17	AXS75258.	*Glycyrrhiza uralensis*	Glycyrrhizin UDP-glucose	C-30

No.	Name	Accession number	Plant species	Substrate	Loci
13	UGT73K1	AAW56091.1	*Medicago truncatula*	Hederagenin, Soyasapogenols B and E UDP-glucose	C-3, 28
14	UGT74AE2	–	*Panax Quinquefolium*	Protopanaxadiol UDP-glucose	C-3
15	UGT74M1	ABK76266.1	*Vaccaria hispanica*	Gypsogenic acid UDP-glucose	C-28
16	UGT94Q2	–	*Panax Quinquefolium*	Ginsenoside Rh2 UDP-glucose	C-3
17	UGTPg1	–	*Panax ginseng*	Protopanaxadiol UDP-glucose	C-3
18	UGTPg100	–	*Panax ginseng*	Ginsenoside RF1, Protopanaxatriol UDP-glucose	C-6
19	UGTPg101	–	*Panax ginseng*	Ginsenoside RF1, Protopanaxatriol UDP-glucose	C-6, 20
20	Pg3-O-UGT1	–	*Panax quinquefolius*	Protopanaxadiol UDP-glucose	C-3
21	GmSGT2	BAI99584.1	*Glycine max*	Soyasapogenol B monoglucuronide UDP-galactose	C-3
22	GmSGT3	BAI99585.1	*Glycine max*	Soyasaponin III UDP-rhamnose	C-3
23	UDPG	–	*Panax ginseng*	Ginsenoside Rd. UDP-glucose	

Table 2.
Overview of plant UGTs in triterpenoid saponins biosynthesis.

3. Biosynthesis of natural products in yeast

3.1 Biosynthesis of flavonoids in yeast

Flavonoids are among the most extensively investigated natural products, which could be divided into several subgroups, including common flavonoids (e.g., galangin, eriodictyol, catechin, quercetin, luteolin, myricetin and cyanidin), isoflavonoids (e.g., genistein) and neoflavonoids (e.g., calophyllolide, isodispar B). Due to their physiological activity and decoded synthesis pathways, the heterologous biosynthesis of flavonoids and their derivatives, have been extensively studied in microbial hosts mostly in *E. coli*. However, high-level flavonoids could also be produced by yeast [11].

Based on yeast platform, 531 mg/L resveratrol production was achieved via the tyrosine pathway directly using glucose and ethanol as substrate in fed-batch fermentation. Through the subsequent pull-push-block strain engineering strategy, more resveratrol production formed via the phenylalanine pathway increased up to 800 mg/L directly from glucose which was the highest titer of resveratrol up to now.

The co-culture system was also developed for flavonoids production. In the collaboration system of *E. coli* and yeast to produce naringenin, *E. coli* provides precursors tyrosine and acetate for yeast to produce naringenin, and finally 21.16 mg/L naringenin was obtained from xylose.

3.2 Biosynthesis of alkaloids in yeast

Alkaloid compounds, especially plant-derived benzylisoquinoline alkaloids and monoterpene indole alkaloids are considered as a valuable source of pharmaceuticals for its anticancer, antiviral and antimalarial activities, et al. In order to replace plant-extracting method, the reconstruction of plant-derived alkaloid biosynthetic pathways in microbes are extensively studied. Though always achieve much lower titers than in *E. coli*, yeast platform was used to produce alkaloid with complex pathway.

By overexpressing 14 known monoterpene indole alkaloid pathway genes and an enhancement of secondary metabolism through overexpression of additional seven genes and deletion of three genes, strictosidine was produced in yeast with a production of 0.53 mg/L. (S)-reticuline (around 80 μg/L), baine (6.4 μg/L) and hydrocodone (0.3 μg/L) have been produced in yeast from simple sugars. Indeed, the titers was low; However, very recently, the total assembly and optimization of the noscapine biosynthetic pathway involving over 30 enzymes in yeast was realized, which was very hard to reconstruct in *E. coli*, and led to a final titer of 2.2 mg/L noscapine using sugars as substrate [12].

3.3 Biosynthesis of terpenes and saponins in yeast

Similar with flavonoids and alkaloids, some simple terpenoids skeletons such as monoterpenoid and diterpenoid, which were produced directly by terpenoid synthase, were mostly studied in *E. coli* for the high activity and easy expression of these terpenoid synthase in *E. coli*. However, in recent years, yeast hosts also attracted more and more attention in these fields.

By expressing geraniol synthase from *Ocimum basilicum*, 0.5 mg/L geraniol was obtained in the engineered yeast. The inefficient supplement of precursors GPP should be responsible for the low production. To improve the final geraniol concentration, an ERG20 mutant was used to block the competitive pathway and the production was improved to 5 mg/L. Based on the inherent GPP and the blocking of the competitive FPP synthesis pathway, other volatile monoterpenes, including sabinene and limonene, were also produced by expressing plant monoterpene synthase in yeast host.

Moreover, the heterologous biosynthesis of diterpenoid by yeast also became a new trend and gained more and more attention. Through introducing diterpenoids synthase and metabolic optimization, several plant diterpenoids including taxadiene and miltiradiene were produced in yeast. With the introducing of the taxadiene biosynthetic pathways, and the optimization of precursors supplement by strengthen the MVA pathway through overexpressing tHMG1 and UPC2-1 (a global transcription factor of MVA), the taxadiene was successfully produce in the engineered yeast and achieved a production of 8.7 mg/L, though the production was lower than that produced in *E. coli* (1 g/L). However, when it involves in more complex skeletons, such as ferruginol and carnosic acid which need further oxidation by CYP450s, yeast displays the huge potential. The membrane located plant CYP450s are hard to functionally expressed in *E. coli*. By co-expressing CYP76AH1 and plant cytochrome P450 reductase (CPR) genes heterogeneously in a miltiradiene producing yeast strain, ferruginol was successfully achieved with a titer of 10.5 mg/L. Based on ferruginol, carnosic acid, a C20 oxidations products of miltiradiene, was produced in yeast by introducing an extra CYP76AK6-8 and these compounds still cannot be produced by *E. coli*.

As an exception, the heterogeneous biosynthesis of triterpenoid and triterpenoid saponins were mostly studied in yeast, for the membrane located plant OSC and CYP450 are challengeable to correctly express in *E. coli*. By overexpressing the

codon optimized OSC from *Glycyrrhiza glabra* designated as β-amyrin synthase (βAS), Zhang et al. obtained a β-amyrin production as high as 138.8 mg/L in the engineered *S. cerevisiae* Sgib [13]. Through co-expressing the liquorice CYP88D6 and CYP72A154 with β-amyrin synthase and CPR genes in yeast, glycyrrhetinic acid was produced with a titer of about 20 μg/L. To boost the glycyrrhetinic acid titer, Zhu et al identified novel CYP450s uni25647 and CYP72A63 with higher activity than CYP88D6 and CYP72A154 by yeast host. With these two CYP450s and a novel liquorice CPR, Zhu et al. reconstructed the GA synthesis pathway in sgib and improved the glycyrrhetinic acid titer up to 18.9 mg/L, which is 1000 folds compared with that in the former study [14]. Zhao et al. overexpressed an efficient CPR (MtCPR1) gene, and reconstructed the galactose regulatory network by knocking out GAL1 and GAL80 while using strong inducible promoters GAL1 and GAL10 to operate CYPP450, MtCPR1, ERG1 and ERG9). Finally, 186.1 mg/L oleanic acid was achieved in yeast, which is the highest concentration in reported literatures [15]. More recently, Yu et al. introduced a high specific α-amyrin synthase in yeast leading to the production of α-amyrin, β-amyrin and lupeol at a ratio of 86:13:1 with the α-amyrin titer of 11.97 mg/L, 5.8-folds of the maximum production reported [16].

Benefit from the yeast hosts, the more complex saponins were also heterologously synthesized. By expressing *Barbarea vulgaris* UGT73C11 in a glycyrrhetinic acid producing yeast strain, Liu et al. realized the de novo synthesis of glycyrrhetinic acid-3-O-monoglucose starting from sample glucose [17]. With the decoding of plant biosynthetic pathways, many other triterpenoid saponins have been successfully synthesized in yeast factories and the high production of ginsenoside Rh2 (2.5 g/L) has highlighted the commercial feasibility of this approach.

Panax ginseng is a famous herb medicine widely used in Asia, which possesses various pharmaceutical activities including anticancer, anti-inflammatory and antiviral activities. These activities are mainly endowed by its triterpenoids saponins, known as ginsenosides [18]. Because of their low content and the long culture cycle of ginseng, the heterologous biosynthesis of ginsenosides in microbial cell factories is drawing more and more attention. To date, various ginsenosides and its aglycones have been successfully produced in *S. cerevisiae* (**Figure 2**), including protopanaxadiol, protopanaxatriol and oleanolic acid, the three main kinds of aglycones, and four kinds of ginsenosides, ginsenosides Rh2, Rg3, RF1 and Rh1. In addition, some unnatural ginsenosides such as compound K and 3β, 12β-Di-O-Glc-PPD have also been synthesized by combination various enzymes from different species.

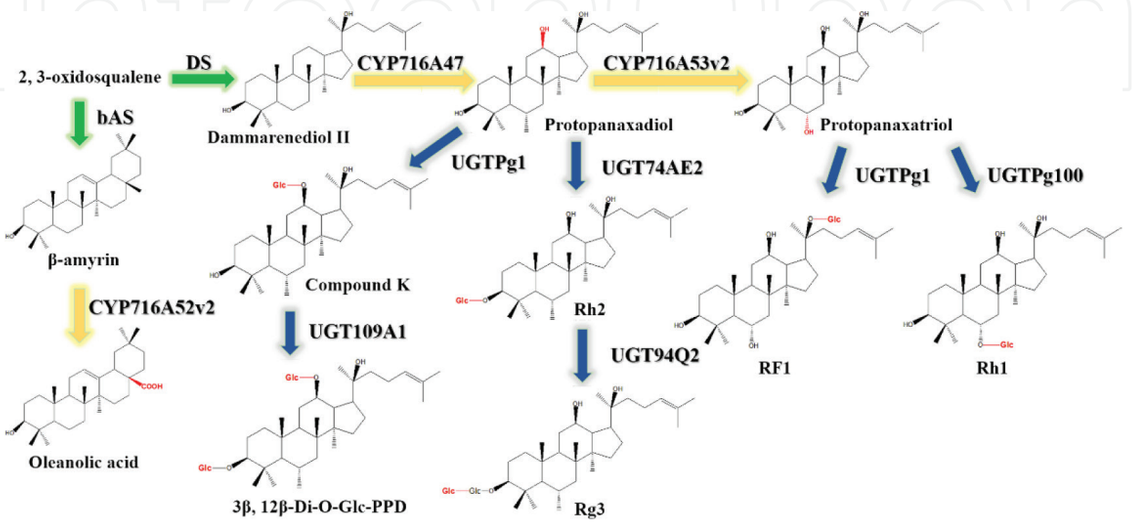

Figure 2.
Scheme for ginsenosides biosynthesized in S. cerevisiae. The green arrows represent OSCs, the yellow arrows represent CYP450s, the blue arrows represent UGTs.

Protopanaxadiol (PPD) is an important starting material for the biosynthesis of ginsenoside, which is synthesized from 2,3-oxidosqualene by dammarenediol-II synthase and CYP450s. Through expressing *P. ginseng* dammarenediol-II synthase (PgDS), *P. ginseng* CYP716A47 and *A. thaliana* CYP450 reductase 1 (AtCPR1), PPD was firstly produced in *S. cerevisiae* [19]. Subsequently, the more complex ginsenosides aglycone protopanaxatriol (PPT) was produced by engineered yeast carrying *P. ginseng* CYP716A53v2 and AtCPR1 which uses PPD as substrate. By the similar strategy of co-expression of *P. ginseng* β-amyrin synthase (PNY1) CYP716A52v2 and AtCPR1, oleanolic acid (OA), a oleanane-type pentacyclic triterpene, was synthesized in *S. cerevisiae* [20]. Although these three ginsenoside aglycones had been successfully synthesized in yeast, the titer was still too low. A truncated version of tHMG1 (3-hydroxyl-3-methylglutaryl-CoA reductase), ERG20 (farnesyl diphosphate synthase), ERG9 (squalene synthase) and ERG1 (2,3-oxidosqualene synthase) were overexpressed to improve the PPD production in *S. cerevisiae*. Together with a codon optimized *P. ginseng* CYP716A53v2, the production of PPD was increased by 262-fold and up to nearly 1.2 g/L. The efficient supplement of PPD made the strain an ideal platform for further tailored ginsenosides biosynthesis [21]. By similar strategies, the PPT production was increased by overexpressing *ERG9*, *ERG1*, *tHMG1* and corresponding CYP450 genes with codon optimization.

The widely studied ginsenosides Rh2 and Rg3 which are synthesized from PPD have been successfully biosynthesized in *S. cerevisiae*. By expressing PgDS, CYP450 system consisting of CYP716A47, AtCPR2 to supply PPD, and the co-expressing of PgUGT74AE2 and PgUGT94Q2 for glycosylation of PPD, Rg3 was heterologously synthesized in *S. cerevisiae*. Combine with the approach of replacing the native promoter of ERG7 with a methionine-repressible promoter (MET3), the production of Rg3 was increased up to 1.3 mg/L. [22] Other type of ginsenosides such as RF1 and Rh1 were synthesized through the co-expression of PgDS, CYP716A53v2, CYP716A47, PgCPR1 and UGTPg100 (or UGTPg1). The production of RF1 and Rh1 reached 42.1 and 92.8 mg/L in *S. cerevisiae*, respectively [23].

It is demonstrated that compound K (CK), generally considered as the metabolite of glycosidases [24], is the main functional form of oral administration of ginsenosides [25]. By the co-expression of PgDS, AtCPR2, CYP716A47 and UGTPg1, CK has already been synthesized in *S. cerevisiae*. Its production was further increased up to 1.4 mg/L by overexpressing tHMG1 and UPC2.1 as well as controlling heterogeneous genes via GAL promoters [26]. By combination of tailoring enzymes from various species, more unnatural ginsenosides could be synthesized in *S. cerevisiae*. Through the combination of plant PgDS, CYP716A47, AtCPR1 and UGT109A1 from *B. subtilis*, 3β, 12β-Di-O-Glc-PPD was produced in *S. cerevisiae*. To further optimize the production, overexpression of tHMG1 and the fusion expression PgDS was carried out, and ERG7 (encoding lanosterol synthase) promoter was replaced with an antisense one. Through these strategies, the production of 3β,12β-Di-O-Glc-PPD was increased up to 9.05 mg/L in the engineered *S. cerevisiae* [27].

Besides ginsenosides, the heterogeneous biosynthesis of other triterpenoids saponins with markedly physiological function also attracted much attention. The natural sweeter mogroside V from S. grosvenorii, which is nearly 300 times sweeter than sucrose, is widely used as a food additive in low-calorie sweet beverages [28]. Through analysis of S. grosvenorii transcriptome data and gene mining, the key genes involved in mogroside V synthesis including cycloartenol synthase (CAS) gene, epoxide hydrolases (EPH) gene, CYP102801, UGT94-289-3 and UGT720-269-1 have been identified. By introducing these enzymes together with squalene synthase (SQS), squalene epoxidase (SQE) and AtCPR1 in *S. cerevisiae*, mogroside V was successfully produced. These advances highlight the possibility to produce natural, noncaloric sweetener mogrosides by engineered yeast.

Saikosaponins are the major pharmaceutical constituents of *B. falcatum*, an important perennial herb widely used in traditional Chinese medicine which exert multiple bioactivities [29]. Currently, two saikosaponins aglycones, 16α-hydroxy α-amyrin and 16α-hydroxy β-amyrin have been synthesized in engineered *S. cerevisiae* through co-expressing CYP716Y1, AtCTR1, CaDDS (dammarenediol synthase from *Centella asiatica*) or GgbAS (β-amyrin synthase from *Glycyrrhiza glabra* in *S. cerevisiae*, respectively [30].

Moreover, by overexpressing lycopene synthetic genes from *Erwinia uredovora*, this ungroomed tetraterpene compound was synthesized by the engineered yeast strain, with a production of 3.3 mg/g CDW. To boost the lycopene production in yeast, Ma et al. overexpressed key genes associated with fatty acid synthesis, TAG production, and TAG fatty acyl composition, and deleted FLD1 to increase lipid-droplet size for more hydrophobic storage space of lycopene, leading to a production of 2.37 g/L, which weighed against that produced in *E. coli* [31]. This indicates the potential of yeast to produce simple terpenoid directly synthesized by terpenoid synthase.

All these progresses indicate the great potential of yeast for heterologous synthesis of natural products, especially for these with complex molecular structure and synthetic pathways such as terpenoids and their saponins. Recently, various kinds of terpenoids and saponins were heterologously produced in yeast, but most of them had a low final concentration far from to instead of plant extracting methods. To boost the production efficiency of engineered yeast strains, various strategies need intensively study.

4. Strategies for boosting biosynthesis of terpenoids in yeast

Although various kinds of natural products could be produced by yeast, many challenges still remain for this approach. The main bottleneck of building an efficient yeast cell factory was that the biosynthetic pathways are not totally elucidated and the poor or disappeared activity of plant enzymes when expressed in yeast. Moreover, the destabilization on the native metabolic flux caused by the heterogeneous pathways could lead to low cell growth and low final products concentration, and the cytotoxicity most of natural products also restrict the use of microbial hosts for producing natural products. Strategies and biotools focused on settling such issues to accelerate the microbial natural products biosynthesis in yeast host have been developed based on omics, metabolic engineering and protein engineering (**Figure 3**).

4.1 Strategies to redesign natural biosynthetic pathways

Unlike some prokaryotic biosynthetic pathway genes, which always locate on a gene cluster, the genes involved in triterpenoid saponins biosynthesis always distributes among the whole genome in plants. Moreover, the expression of these genes generally needs intricate inducible conditions, which increase the difficulty to elucidate triterpenoid saponins biosynthetic pathways.

Benefit from the rapid progress of sequencing technology, genome and transcriptome of many medicinal plants have been sequenced, and the information has been publicly available online (http://medicinalplantgenomics.msu.edu). The analysis of the genome and transcriptome date facilitates the prediction of genes involved in the targeted compound biosynthesis. Through the comparison of the transcriptome information between plants or tissues with high- and low-production of the target compounds, several key genes could be predicted. For example,

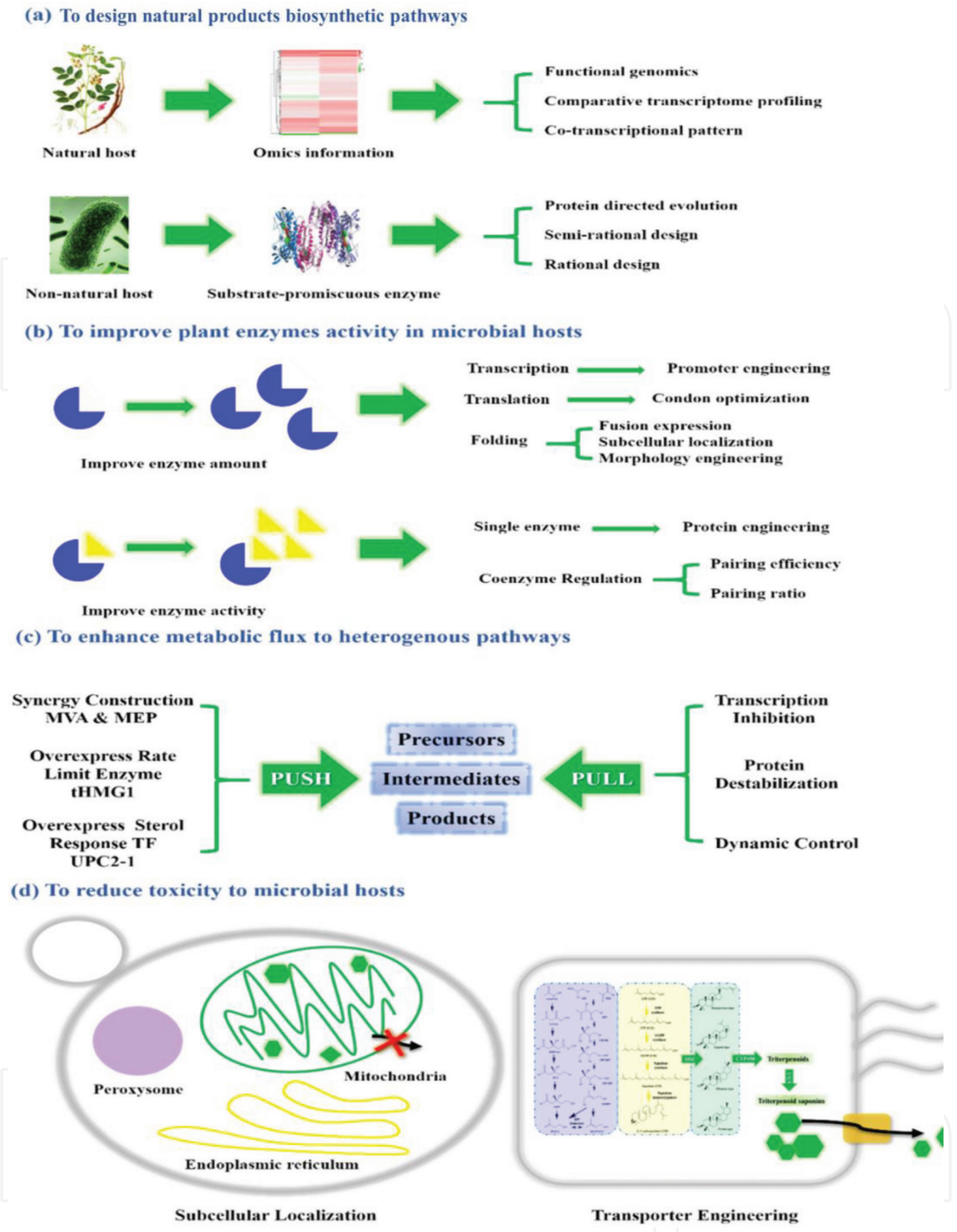

Figure 3.
Scheme for strategies used to advance biosynthesis of triterpenoid saponins in microbial hosts. (a) Strategies to design triterpenoid saponins biosynthetic pathways; (b) Strategies to improve plant enzyme activity in microbial hosts; (c) Strategies to enhance metabolic flux in microbial hosts; (d) Strategies to reduce toxicity to the hosts.

through comparing the transcriptome data between high- and low-producing varieties, the genes including bAS1, CYP716A79 and CYP716A78 that involved in quinoa saponins biosynthesis were targeted from *Chenopodium quinoa*. The predicted genes were then functionally identified by expressing in yeast [32]. By comparing the transcriptome data between ethyl alcohol and methyl jasmonate elicited conditions, key genes involved in maesasaponins biosynthesis including bAS, CYP716A75 and CYP87D16 from *Maesa lanceolate* were targeted and were subsequently identified by expressing in yeast host [33]. Depending on the comparison

of the transcriptome data between varieties with different phenotype, key genes involved in target compound biosynthetic pathways can be predicted, and be further identified by functionally expressed in yeast. Generally, the expression patterns of genes involved in the same biological process are strongly correlated. Thus, genes co-expression analysis was applied to predict the functions of unidentified genes. Based on this strategy, CYP716A12, CYP93E2, CYP72A61v2 and CYP72A68v2 from *M. truncatula* have been functionally identified [34].

Besides mining enzymes from native host, the application of the substrate-promiscuous enzymes turned out to be an alternative approach to reconstruct the target compounds biosynthetic pathways. For instance, the nonnative substrate-promiscuous glycosyltransferase Bs-YjiC from *B. subtilis* 168 was used for the synthesis of ginsenoside Rh1 and some unnatural ginsenosides in *S. cerevisiae* [35]. Other substrate-promiscuous enzymes like UDP-glycosyltransferase UGT109A1, was also used to produce the unnatural ginsenosides 3β, 12β-Di-O-Glc-PPD, 3β,12β-Di-O-Glc-PPT, 3β,20S-Di-O-Glc-DM, and 3β-O-Glc-DM.

The low final concentration of the synthesized compounds is always caused by the poor enzyme activity on the unnatural substrate. Protein evaluation, which could improve the catalytic characteristic involving "substrate specificity" of the substrate-promiscuous enzymes has been developed for the specific decoration of natural and unnatural substrates. One example is the engineering of the substrate-promiscuous UDP-glucose sterol glucosyltransferase UGT51 from *S. cerevisiae*. UGT51 can glycosylate a series of structural analogues, including pregnenolone, cholesterol, ergosterol, sitosterol, diosgenin, estradiol, PPD and PPT. Because of the wide substrate spectrum, UGT51 has been applied for the biosynthesis of ginsenosides Rh2 with the co-expression of PgDS, CYP716A47 and AtCPR1 in *S. cerevisiae*. However, only trace amount of Rh2 was synthesized. To improve the enzyme activity, a semi-rational design strategy was developed based on the crystal structure of UGT51 (PDB code: 5gl5). The best UGT51 mutant gained an 1800-fold higher catalytic efficiency (kcat/Km) in converting PPD to ginsenoside Rh2 in vitro and the Rh2 production reached up to 300 mg/L in vivo [35]. Using non-native promiscuous enzymes to reconstruct the natural products biosynthetic pathways is an efficient strategy to achieve the heterogeneous synthesis of triterpenoid saponins in yeast, especially for that without integrated illuminated pathways.

4.2 Strategies to improve plant enzyme activity

Plant CYP450s are indispensable enzymes for the C–H bounds oxidation of triterpenoid skeletons. However, heterogeneous expression of plant CYP450s in yeasts hosts usually exists problems as low expression level, poor catalytic efficiency or even incorrect folding structure. Plant CYP450s involved in triterpenoids synthesis are membrane-bound oxidase enzymes, which anchor in the endoplasmic reticulum (ER) of plants cells and requires electrons transferred by CYP450 reductase. Although CYP450 is essential for the hydroxylation of C–H bounds and can further oxidize the alcohol products to aldehyde and acid, it always shows poor activity on such substrates. As a result, it is challenging to establish high-yield triterpenoid saponins in yeast cell factories, and improving the plant CYP450s is essential to improve the situation. Currently, many strategies have been developed to improve the expression level and regulate the pairing efficiency of CPRs to plant CYP450s in microbial hosts.

Codon optimization and application of a strong promoter are the most common strategies to improve enzyme expression level in heterologous hosts and it is also effectively used to improve plant CYP450s expression level in microbial hosts [36]. Chimeric protein has been used to correct the folding of plant CYP450s in microbial

hosts. As plant CYP450s are anchored in ER membrane, the activity can be improved by replacing the native N-terminal sequence with ER-membrane bound proteins of yeast to facilitate correctly folding and anchoring. Protein directed evolution was also applied to enhance the activity of plant enzymes. In consideration of that the enlargement of ER would provide more room for the ER-located CYP450s and CPRs leading to higher protein abundance, a novel ER morphology engineering strategy is developed. Through the deletion of PAH1 gene encoding phosphatidic acid phosphatase, the ER membranes of *S. cerevisiae* was tremendously expanded, which accelerated the expression of CYP450s including CYP716A12, CYP72A67 and CYP72A68, and ultimately leading to increased production of triterpenoid and triterpenoid saponins. Approaches like codon optimization, Chimeric protein, protein directed evolution and ER enlargement have been used to improve CYP450s expression level in heterologous hosts.

The pairing efficiency of CYP450s and CPRs plays an important role for the catalytic activity of CYP450s. Mining novel CPRs is a straightforward way to improve the CYP450 activities as different CPRs has different pairing efficiency with CYP450s. For example, the CPR from *M. truncatula* was proved to be the most efficient one pairing with CYP716A12 among all the tested CPRs from *G. uralensis*, *Lotus japonicus* and *A. thaliana*, and boosted OA biosynthesis in yeast [15]. Moreover, the co-expression of CYP72A63 and/or uni25647 with GuCPR1 from *G. uralensis* showed higher activities than the co-expression with other CPRs from *M. truncatula*, *A. thaliana*, or *L. japonicus*. The using of GuCPR1 resulted in boosted GA production in yeast [14]. Besides the using different CPRs, ratios between CYP450 and CPR also contribute to the CYP450 activities. Fine-tuning the ratios between CYP450 and CPR is an important strategy to improve their pairing efficiency. For example, with the best ratio of 5:9 between CYP716Y1 and CPR1 from *A. thaliana*, the 16-α-hydroxy amyrin production was significantly increased [30]. These results indicate that improving the pairing efficiency by efficient CPRs and proper pairing ratio between CYP450 and CPR can improve the targeted compound production in yeast.

4.3 Strategies to enhance metabolic flux

The plant natural products biosynthetic pathways always include multiple steps. When introduced in yeast, the heterogeneous pathways would intensively interact with the native metabolic network, by means of competing substrates and co-factors as well as metabolites reverse influence. The disturbance will restrict the targeted compound production. Therefore, balancing metabolic flux distribution between heterologous pathways and native metabolic networks plays an important role in promoting the production of targeted compound.

Enhancing the precursors supplement to the targeted pathway is a straightforward strategy to enhance natural products production. As demonstrated, the five-carbon building blocks IPP and DMAPP are naturally synthesized through either eukaryotic MVA or prokaryotic MEP pathway in microorganisms. The combination of MVA and MEP pathways in one host could take advantages of both pathways and lead to more efficient precursor supplement for terpenoids. Through introducing a heterogeneous MVA pathway, 27.0 g/L amorphadiene was achieved in *E. coli*. Besides, the production of other terpenoids such as valerenadiene (62.0 mg/L), isoprene (24.0 g/L), and lycopene (47.0 mg/L) were also successfully increased by the synergy of the MEP and the MVA pathway in *E. coli*. In view of the availability of this strategy, the combination of MVA and MEP pathway have been developed in yeast. In addition to the cooperation of the MEP and the MVA pathways, taking full advantage of precursors synthesized in different organelles was helpful to boost

the terpenoids producing. By improving the utilization of acetyl-CoA through the simultaneously introducing dual MVA pathways located in cytoplasmic and mitochondrial of yeast, 2.5 mg/L isoprene was obtained [37]. Moreover, HMG-CoA reductase catalyzing the conversion of HMG-CoA to mevalonate is a rate-limiting factor of MVA pathway. The HMG-CoA reductase is feedback regulated due to its N-term transmembrane sequence, so the truncated version (N-term truncated) of HMG-CoA reductase (tHMG1) is intensively used to strengthen the precursor supplement. Globe transcriptional regulation by overexpressing UPC2-1, which is a global sterol regulatory element to induce sterol biosynthetic genes expression, is another effective method to improve the MVA flux globally [38].

Decreasing the metabolic flux of competing pathways is efficient to strengthen the flux to targeted pathway. However, in most cases, directly deletion of the enzymes involved in the competing could lead to lethality, for many of the genes are essential to the hosts. Therefore, decrease the metabolic flux to the competing pathways by down-regulation of key enzymes is a proper approach to strengthen the final production. Generally, the cellular protein concentration is regulated by transcription, RNA degradation, translation and protein degradation. The application of weaker promoters is the most commonly used strategy to down-regulate the transcriptional level of key genes. To decrease the sterol synthesis and redirect the metabolic flux to the β-amyrin synthesis pathway in yeast. ERG7 (lanosterol synthase gene) promoter was replaced by a methionine repressible promoter (PMET3). Moreover, in order to improve the α-santalene accumulation in yeast, the native promoter of ERG9 (squalene synthase gene) was replaced by PCRT3, the copper repressible promoter and PHXT1, a low concentration glucose repressible promoter which resulted in decreased metabolic flux to ergosterol synthesis and increased α-santalene production [39]. Recently, dynamic protein degradation was developed to weaken the competing pathways. Depending on the ER-associated protein degradation system, the cytosolic proteins can be degraded when attached by a PEST sequence. As a result, the strategy of using the G1 cyclin PEST sequence as a degradation degron to label the cytosolic term of squalene synthase was developed for the production of trans-nerolidol. Once labeled by degron, the squalene synthase will be degraded dynamically, resulting in enhanced sesquiterpene trans-nerolidol production. By the similar strategy, farnesyl pyrophosphate synthetase was labeled by a designed N-terminal degron on the N-terminus, which increased the titer of monoterpene linalool [40].

4.4 Strategies to reduce toxicity to the hosts

Natural products always exhibit cytotoxicity to the microbial hosts, leading to decreased cell growth and finally impair the production. In order to solve these problems, various strategies were developed including two-stage fermentation, pathway compartmentalization and transporters mediated compound secretion. In order to alleviate the negative influences on cell growth, the fermentation course is divided into two stages. In first fermentation stage, heterogeneous pathway keep silence and cells grow fast with precursor accumulated, while in the second stage, target pathway would be induced to produce the target compounds [41]. In addition to the traditional two-stage fermentation, the organelles including mitochondria, peroxisome and vacuole were also used to compartmentalize the heterogeneous pathways. Because the integrated membrane structure, these organelles are relatively independent from the cytoplasm, which could prevent the toxic precursors and products from distributing in cytoplasm to disrupt cell growth. Furthermore, subcellular compartmentalization of target biosynthetic pathways can concentrate the substrates, intermediates and enzymes in a more narrow space, which can improve the reaction efficiency of enzymes. Through locating the

amorpha-4,11-diene biosynthetic pathways in the mitochondria of yeast, the amorpha-4,11-diene production increased by 63% compared with locating in the cytosol. By this strategy, the precursor FPP was restricted in mitochondria by the membrane structure, which reduced the loss of FPP. Using similar strategy, the valencene biosynthetic pathway was reconstructed into the mitochondria of yeast resulting in eight-fold increase of valencene production. Besides the mitochondria, peroxisome was also used to compartmentalize the heterogeneous pathways, by introducing the lycopene synthesis pathway in peroxisome, lycopene production was improved up to 73.9 mg/L in Pichia pastoris [42]. Therefore, subcellular compartmentalization was a pioneering strategy to reduce products cytotoxicity to the microbial hosts.

Another strategy to reduce the inner cytotoxicity of natural products is to secrete these compounds outside the cell automatically. To achieve this goal, transporters were taken into account, for their significant contribution of transporting the products to the extracellular space. Due to the rarity of transporters that possess the ability to transport the complex natural products, transporter engineering has been developed to improve the situation. For example, through protein engineering, one variant of AcrB from AcrAB-TolC efflux pump can effectively improve the α-pinene efflux out of the *E. coli* cell. However, because of unclear of the transport mechanisms and lacking of crystal structures, the application of transporters in natural products is still limited.

In this chapter, the biosynthetic pathways of natural products and their reconstruction in yeast cell factories were systematically summarized. The strategies developed to increase natural products productivity in yeast were also discussed including protein engineering, metabolic engineering, subcellular localization and fermentation control. With these endeavors, the engineered strains can produce these compounds in different levels. These achievements indicate yeast a promising chassis for the heterogeneous biosynthesis of natural products.

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