CellPress

Special Issue: Industrial Biotechnology

Review

Production of Useful Terpenoids by Higher-Fungus Cell Factory and Synthetic Biology Approaches

Han Xiao[1,*] and Jian-Jiang Zhong[1,*]

Higher fungi with greater than 70 000 species are regarded as a rich source of various natural compounds including terpenoids, the production of which represents a wide range of interest in pharmaceutical and healthcare industries. This review summarizes the current knowledge of terpenoids synthesized by higher fungi, and highlights the current state-of-the-art regarding genetic manipulation of higher fungi. As the focus, this article will discuss the most recent approaches enabling native hosts and heterologous microbes to efficiently produce various terpenoids, especially with regard to the construction of 'smart' higher-fungus cell factories. The merits and demerits of heterologous versus native hosts as cell factories will also be debated.

Higher Fungi and Their Useful Terpenoids

Higher fungi (also known as macrofungi), including divisions of Ascomycetes, Basidiomycetes, and Deuteromycetes, can form easily observed and collected sporocarps as a result of septate hyphae development. They have been recognized as an abundant and important group of organisms to humans for centuries. These fungi are of particular interest not only because of their importance as food resources but also as traditional medicines [1]. For survival in unfavorable environments, higher fungi are able to synthesize diversified functional secondary metabolites. Until now, many bioactive compounds from higher fungi have been isolated, identified, and characterized with various biological and pharmacological activities, including terpenoids, heterocyclics, polysaccharides, and polyketides [2].

Displaying a wide range of biological activities, terpenoids, which contain sesquiterpenoids, diterpenoids, and triterpenoids, are the most attractive metabolites among the myriad of natural products from higher fungi (Table 1). Irofulven (or 6-hydroxymethylacylfulvene), an analog of the sesquiterpenoid illudin S, was able to induce apoptosis in human cancer cell lines by boosting the immune system. Although it exhibited encouraging results in shrinking malignant solid tumors and drug-resistant cancers during Phase I clinical trials, irofulven had side effects of retinal damage and visual disturbance in Phase II clinical trials [3,4]. Diterpenoid pleuromutilin and its derivatives selectively inhibited bacterial protein synthesis through interaction with the 50S subunit of prokaryotic ribosomes [5]. Derivatives of pleuromutilin, tiamulin, and valnemulin, with improved activity against bacteria and solubility in water, have been developed as antibiotics for veterinary use; and another derivative retapamulin was approved as an antibacterial agent for human use by the FDA, USA in 2007 [6], which is marketed as an ointment under the brand

Trends

Higher fungi are the most plentiful producers for natural products. Those natural compounds including terpenoids have a wide range of bioactivities, which are significant to pharmaceutical and healthcare industries.

Higher fungi are strong candidates for the production of native natural products, owing to their inherently tolerant and suitable expression systems.

Owing to a mysterious genetic background and immature genetic manipulations, higher fungi have long been overlooked by the academia and industrial community. With the help of different levels of 'omics' investigations and the development of genetic manipulation tools, the construction of 'smart' higher-fungus cell factories for useful natural product production (e.g., terpenoids) is believed to be a highly desirable and promising research direction.

[1]State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic and Developmental Sciences, and Laboratory of Molecular Biochemical Engineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai, 200240, China

*Correspondence: smallhan@sjtu.edu.cn (H. Xiao) and jjzhong@sjtu.edu.cn (J-J. Zhong).

 http://dx.doi.org/10.1016/j.tibtech.2015.12.007

Table 1. Terpenoids from Higher Fungi and Their Bioactivities

Terpenoids	Higher Fungi	Bioactivities	Refs
Sesquiterpenoids			
Illudins	*Omphalotus olearius*	Antitumor, antimicrobial	[76]
3α,6β-Dihydroxycinnamolide	*Inonotus rickii*	Anticancer	[77]
Hirsutane-type	*Stereum hirsutum*	Antimicrobial and antitumor	[78]
Enokipodins C–D, E–J	*Flammulina velutipes*	Antimicrobial, Antifungal	[79,80]
Nambinones A–C	*Neonothopanus nambi*	Anticancer	[81]
Diterpenoids			
Pleuromutilin	*Clitopilus passeckerianus*	Antimicrobial	[82]
Secoscabronine M	*Sarcodon scabrosus*	Anticancer	[83]
Striatoids A–F	Cyathus striatus	Neurotrophic activity	[84]
Cyathins D–H	*Cyathus africanus*	Anti-inflammatory and cytotoxic	[85]
Neosarcodonin A–C	*Sarcodon scabrosus*	Anti-inflammatory	[86]
Triterpenoids			
Ganoderic acids	*Ganoderma lucidum*	Antitumor, anti-HIV, antimicrobial, antimetastasis, antioxidation, etc.	[87]
Lucidenic acids	*G. lucidum*	Anticancer	[88]
Lanostane-type	*Naematoloma fasciculare*	Anticancer	[89]
Ganoboninketals A–C	*Ganoderma boninense*	Anticancer	[90]
Cattienoids A–C	*Tomophagus cattienensis*	Antitumor	[91]

names Altabax and Altargo. Additionally, pilot animal studies using some of these bioactive terpenoids have also shown their promising perspective. For example, the triterpenoid ganoderic acid-T (GA-T) induces mitochondria-mediated apoptosis in cancer cells. In animal studies, it inhibited solid tumor growth in nude mice and enhanced the sensitivity of the first-line anticancer drug, doxorubicin [7]. Because terpenoid metabolites are generally of complicated chemical structures and difficult to be chemically synthesized, biosynthesis of terpenoids by cell factories (Box 1) of higher fungi has received increasing attention to meet the huge requirement for (pre)clinical study and large-scale industrial production. For instance, the higher fungus *Ganoderma lucidum* is used for production of anticancer and antimetastasis GAs. Key enzymes in the GA biosynthetic pathway were overexpressed (e.g., [8]), and various fermentation strategies including a new two-stage cultivation mode were developed to achieve a high GA yield [9]. In addition to the endogenous production system by native hosts, synthetic biology approaches, including the *in vitro* synthetic enzymatic pathway [10,11] and *in vivo* heterologous production by genetically favorable hosts such as *Escherichia coli* and *Saccharomyces cerevisiae* [12,13], are recently regarded as interesting alternatives to original production hosts or for producing non-natural products. For example, biosynthesis of the precursor of antimalarial drug artemisinin, artemisinic acid, which was originally produced by *Artemisia annua*, can be achieved in *E. coli* and *S. cerevisiae* at a very high titer of 25 g/l via the introduction of native genetic components and optimization of the fermentation process [14,15]. In this review, first, the current knowledge on the biosynthesis of terpenoids by higher fungi will be introduced, and the development of genetic engineering of higher fungi will be highlighted. Then, typical examples with regard to the production of terpenoids by higher fungi will be shown. As an alternative and promising choice, a synthetic biology approach to produce terpenoids is consequently discussed. Finally, perspectives on how to further improve terpenoid production will be provided.

Box 1. Cell Factory

Microbial transformation has been exploited by humans since ancient times, especially for early fermented food and drinks. The birth of genetic engineering in the late 1970s and its further technological development have provided molecular tools to engineer and produce heterologous proteins in various microorganisms including bacteria, yeast, and fungi, and thus largely extended their manufacturing capabilities. The so-called modern biotechnology uses microbial cells more directly to produce amino acids, enzymes, proteins, antibiotics, fuels, drugs, food, and biomaterials of economic interest; therefore, currently the cell factory concept is more alive than ever. The contribution of systems biology and synthetic biology principles, metabolic and protein engineering, and 'omics' technologies permits the full exploitation of microbial cells from such an interdisciplinary approach. The cell factory concept is completely structured through the examination of the metabolic capabilities of producer cells in close relationship with the nature and features of the product and the bioproduction process itself. The consequent identification of bottlenecks during production and the cellular responses triggered under this situation reveal the integrated nature and complexity of the biosynthesis machinery and quality control as well as its connections with mechanisms coping with stresses at cellular and population levels. On the basis of these findings, scientists can now better approach improved strategies to adapt microbes to new production requirements for both natural and engineered products. In such a scenario, cells are modified as a 'smart' plant (cell factory) for product biomanufacturing.

Terpenoid Biosynthesis by Higher Fungi

According to the number of five-carbon isoprene units in the scaffolds, terpenoids are classified into hemiterpenes (C5, one isoprene unit), monoterpenes (C10, two isoprene units), sesquiterpenes (C15, three isoprene units), diterpenes (C20, four isoprene units), triterpenes (C30, six isoprene units), tetraterpenes (C40, eight isoprene units), and polyterpenes [$(C5)_n$, n could be 9–30 000). Among these terpenoids, sesquiterpenes, diterpenes, and triterpenes are most frequently identified from higher fungi [16].

Five-carbon intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are common precursors of terpenoids, which are synthesized via the mevalonate pathway in fungi [17]. In this pathway, two molecules of acetyl-coenzyme A (acetyl-CoA) undergo a condensation to yield acetoacetyl-CoA. Then, acetyl-CoA is added to acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is catalyzed by HMG-CoA synthase. Mevalonic acid is further generated by HMG-CoA reductase. This six-carbon mevalonic acid is transformed into the five-carbon IPP after a series of reactions, namely a two-step phosphorylation of the primary alcohol, further phosphorylation of the tertiary hydroxyl, followed by decarboxylation and loss of phosphate. IPP is isomerized to generate DMAPP. Adding IPP to its isomer DMAPP generates geranyl diphosphate (GPP). Condensation of GPP with additional IPP units forms larger prenyl diphosphates – farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) (Figure 1).

Successive head-to-tail 1′-4 condensation of one to three IPP extender units to DMAPP gives rise to sesquiterpenes and diterpenes, respectively. A series of FPP cyclizations, which are catalyzed by sesquiterpene synthases, generate C15 hydrocarbon scaffolds of diverse sesquiterpenoids, such as antitumor illudin [18] (Figure 1). A total of 11 putative sesquiterpene synthase genes were identified from the genome of the illudin-producing mushroom *Omphalotus olearius*. Among these genes, *omp1*, *omp6*, and *omp7* are located in biosynthetic gene clusters, suggesting their important function in illudin biosynthesis [19]. Cyclization of GGPP, which is catalyzed by diterpene synthases, yields C20 scaffolds of diterpenoids. A biofunctional diterpene synthase is responsible for the cyclization during biosynthesis of labdane-related diterpenoid pleuromutilin, which has strong antibacterial activity against Gram-positive bacteria [20] (Figure 1). A bicyclic 6-6 copalyl- or 5-6 diphosphate was yielded via the N-terminal class II domain-mediated cyclization, while the final product was produced by the C-terminal class I domain-catalyzed cyclization [16].

By contrast, longer chain C30 triterpenes are formed by 1′-1 head-to-head dimerizations of two FPP molecules. After formation of C30 squalene via dimerization of two FPP molecules, C30–2,3-oxidosqualene is generated from oxygenation of C30 squalene catalyzed by

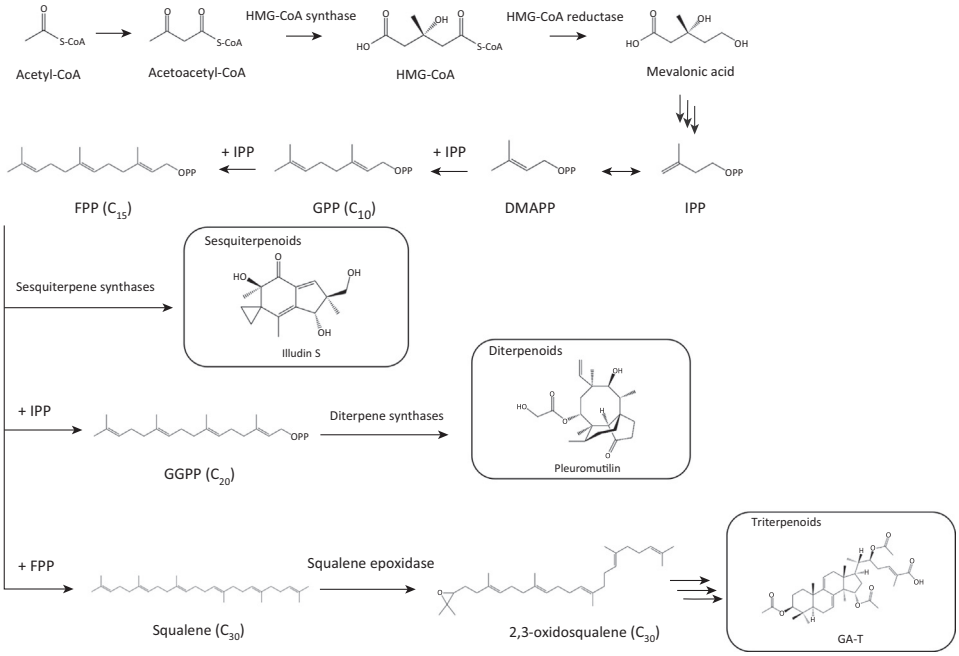

Figure 1. Overview of Terpenoid Biosynthesis in Higher Fungi, in which the Precursors IPP and DMAPP are Synthesized via the Mevalonate Pathway. Abbreviations: Acetyl-CoA, acetyl-coenzyme A; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate.

squalene epoxidase, which is believed to be the skeleton of various triterpenoids (Figure 1). Owing to significant biological activities, GAs are the best known fungal triterpenoids produced by *G. lucidum*, a famous traditional Chinese medicinal herb, of which lanosterol is the most direct precursor [21]. To form different GAs, lanosterol subsequently undergoes postmodifications triggered by different enzymes, mainly cytochrome P450s (CYP450s). A possible biosynthetic pathway of GAs is illustrated here according to literature information (e.g., [22]) (Figure 2).

Genetic Manipulation of Higher Fungi

Genetic manipulation of higher fungi provides a powerful tool to further elucidate terpenoid biosynthesis and to increase the yield of target compounds. To fulfill comprehensive manipulations, platform technologies such as genetic transformation, gene overexpression, deletion, and knockdown are indispensable. Their current status and development are summarized as follows.

Genetic Transformation

The important premise of genetic manipulation is an efficient gene transformation system to deliver extraneous DNA fragments. So far, no plasmid was found to be stably maintained in higher fungi, and extraneous DNA fragments were accordingly integrated into the chromosome for stable expression. Hitherto, polyethylene glycol (PEG) transformation, *Agrobacterium tumefaciens*-mediated transformation (ATMT) and electroporation transformation have been the most commonly used transformation systems in higher fungi.

For PEG transformation, around 80–180 transformants could be obtained per μg of DNA per 10^7 protoplasts in *Pleurotus ostreatus* [23], *G. lucidum* [24], and *Lentinus edodes* [25], respectively. With this method, genes were randomly integrated into the chromosome in the form of multiple copy. By contrast, ATMT allows single copy DNA integration. In a recent study, 10–15

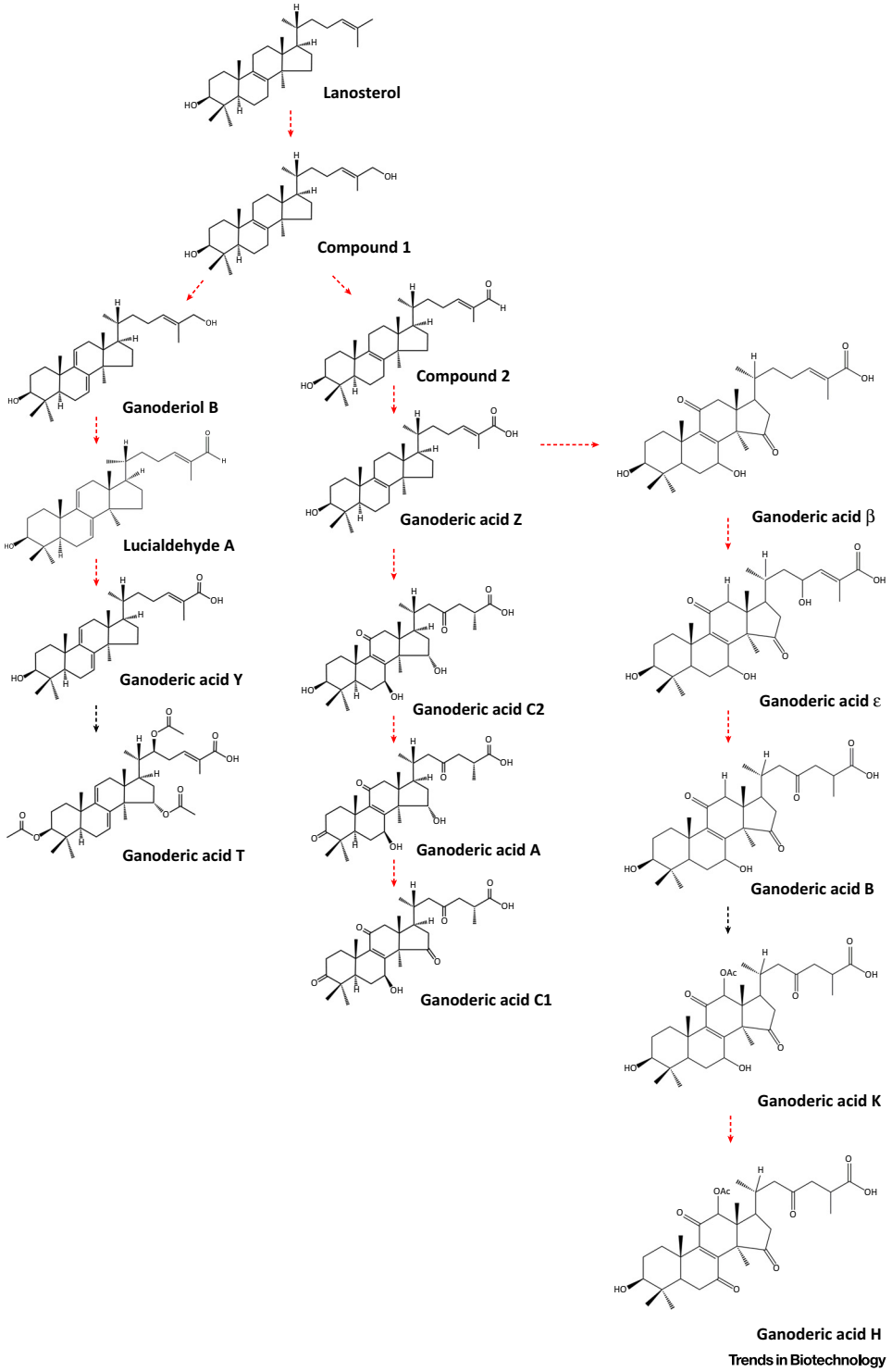

Figure 2. Putative Biosynthetic Pathways of Ganoderic Acids (GAs) from Lanosterol (Adapted from [2]). Dashed red arrows indicate reactions that are catalyzed by CYP450s. Abbreviation: CYP450, cytochrome P450.

transformants per 10^7 protoplasts were obtained in *G. lucidum* by the ATMT method [8]. Unlike dealing with protoplasts, whose preparation is tedious and requisite by both PEG transformation and ATMT, electroporation transformation was developed to transform DNA into basidiospores or mycelial fragments of higher fungi. With the rapid process of preparing basidiospores, efficiency could be achieved as 30–150 transformants per μg DNA in *L. edodes* [26].

Selective Marker

Currently, drug resistance markers are predominant selective markers for the selection of positive transformants in higher fungi. For example, the introduction of the hygromycin B phosphotransferase (*hph*) gene derived from bacteria permits resultant cells growing in the presence of hygromycin. A mutant *sdhB* gene, encoding an iron–sulfur protein subunit of succinate dehydrogenase, was proven to be a suitable carboxin resistance marker in *G. lucidum* [8]. In future, with the wide application of various genetic tools in higher fungi, more and more selectable markers (e.g., nutritional markers) may be available in higher-fungi chassis cells.

Gene Overexpression

In higher fungi, gene overexpression is mainly achieved by increasing the transcriptional level via using either endogenous or heterogeneous promoters. Using the homogeneous *gpd* promoter to drive the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), twofold improvement of GA biosynthesis was attained in the mutant compared with its wild-type *G. lucidum* cells [8]. In another study, increased production of β-glucans was reached in *P. ostreatus* through replacing the endogenous β-glucan synthase promoter with the *gpd* promoter from *Aspergillus nidulans* [27]. Besides, heterologous gene expression can be greatly enhanced in the enoki mushroom *Flammulina velutipes* by employing polycistron containing the viral 2A cleavage sequence. The viral-derived 2A peptides were able to be properly self-cleaved in *F. velutipes* and consequently adopted to connect multiple copies of heterologous protein in one expression cassette. Thus, this strategy can be used for expressing multiple copies of a single gene as well as multiple genes in a single reading frame [28].

Gene Deletion

Strategies of gene deletion in higher fungi are mostly based on homologous recombination (HR). Owing to the inherent low HR efficiency, gene inactivation was only reported in *Schizophyllum commune* with an efficiency of 3.25% [29]. To efficiently screen transformants with ectopic integration, another gene deletion method was developed in this strain. The helper vector pDelcas was constructed with a series of restriction sites, allowing directional cloning of the homologous flanking regions at both ends of the nourseothricin resistance cassette. Besides, a phleomycin selective marker was contained on the backbone plasmid, enabling rapid transformant screening in *S. commune* [30]. Deletion of *ku80*, which is responsible for the non-homologous end-joining system for DNA repair, further increased the deletion efficiency, but with the sacrifice of 100-fold reduction of transformation efficiency in *S. commune* [31]. Likewise, gene disruption frequency was enhanced by inactivation of the *ku70* gene in *Coprinopsis cinerea* [32].

Gene Knockdown

As a useful technology, gene knockdown plays a significant role in studying the regulation of metabolic flux and characterization of gene functions, particularly in cases where essential genes are investigated. RNA interference (RNAi) is the predominant gene knockdown technique in higher fungi, which inhibits gene expression by promoting mRNA degradation. To characterize the function of the squalene epoxidase (*erg1*) gene in *Hypholoma sublateritium*, silencing of *erg1* expression was finally chosen since the expected gene deletion mutant cannot be obtained. A 800- or 1600-bp fragment of *erg1* in the 3′-5′ orientation driven by the gdhA promoter and transcriptional terminator of *Agaricus bisporus* was contained in the antisense RNA expression cassette. Integration of this cassette into chromosome successfully reduced

erg1 expression by 50%, and decreased the production of clavaric acid (an antitumor triterpenoid) and ergosterol-dependent phenotype, indicating the function of the gene in clavaric acid biosynthesis [33].

Bioproduction of Terpenoids

Given the significant roles that natural terpenoids play in the biopharmaceutical and healthcare industries, the bioproduction of terpenoids at high yield, productivity, and titer is extremely important. Although many research contributions have been made in developing genetic manipulation tools in higher fungi, cases related to improving endogenous terpenoid production are very limited. For instance, very few investigations have been performed for bioactive sesquiterpenoid production. Besides, until now the studies for terpenoid production have been mostly focused on optimizing fermentation processes. Meanwhile, exogenous biosynthesis of terpenoids via a synthetic biology approach is another interesting strategy. With the elucidation of biosynthetic pathways, a few important sesquiterpenes, diterpenes, and triterpenes are to be gradually synthesized heterologously by genetically tractable hosts.

Endogenous Production of Terpenoids by Higher-Fungus Cell Factories

Pleuromutilin is a famous antibacterial diterpenoid originally synthesized by mushrooms *Clitopilus passeckerianus* and *Pleurotus mutilis*. Initially, chemical mutagenesis of *C. passeckerianus* and optimization of its fermentation medium was adopted for screening mutants with higher pleuromutilin production. As a result, a mutant strain Cp76, exposed to 0.15 mg/ml of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), was screened out and able to produce 67.4% higher pleuromutilin in the locally formulated medium in 2.6 l of fermentor than in the original medium composition [34]. Later, batch kinetics, modeling, and fermentation conditions of pleuromutilin by another higher-fungus *P. mutilis*, were systematically investigated. In batch cultivation at pH 7.5 and temperature of 27 °C, the Luedeking–Piret equation applies to the pleuromutilin production kinetics by *P. mutilis* [35]. Another investigation showed that using constant glucose concentration during fed-batch cultivation led to high pleuromutilin production at 10.15 g/l and 0.053 g/l per hour in *P. mutilis* fermentation [36]. In a more recent study, higher content of pleuromutilin was obtained by enhanced expression of CYP450 family 1 in *C. passeckerianus* [37].

As a type of highly oxygenated lanostane-type triterpenoid, many GAs and their derivatives have been proven to have anticancer and other pharmacological activities [38]; therefore, their bioproduction has attracted plenty of interest from both academia and industry. In one of our previous studies, a two-stage shaking–static fermentation concept was proposed to promote GA production. This new fermentation mode resulted in high production of total GAs at 3.19 mg/100 mg of dry cell weight (DCW), while only 1.36 mg/100 mg of DCW of total GAs could be obtained in traditional fermentation systems [39]. In a bioreactor submerged fermentation process, by optimization of various fermentation conditions, a GA production titer of 754.6 mg/l was achieved by a strategy of pH shift and dissolved oxygen tension (DOT) shift integrated with lactose feeding [40]. Addition of Ca^{2+}, Na^{+}, Mn^{2+}, phenobarbital, and limitation of nitrogen source could also enhance GA accumulation, among which addition of Ca^{2+} at 10 mM showed the most significant effect, resulting in production of 71.12, 1.91, 11.94, 2.33, and 3.03 mg/g DCW of total GAs, individual GA-MK, -T, -S, and -Me, respectively, in two-stage fermentation processes [41–44]. Furthermore, a new three-stage light irradiation strategy for GA production was also developed with 466.3 mg/l of maximal GA production [45].

In addition to optimization of the fermentation process, studies on metabolic engineering of *G. lucidum* for enhancing GA production were also performed. Owing to the difficulty of gene deletion in higher fungi, strategies were primarily focused on overexpression of key biosynthetic genes. For instance, overexpression of HMGR gene led to twofold increase in GA content (reaching 30.69 mg/g DCW) [8], and overexpression of squalene synthase gave rise to

production of individual GA-MK, -T, -Me, and -S at 16, 40, 43, and 53 μg/100 mg DCW, respectively, which were 2.86-, 2.67-, 1.95-, and 1.25-fold of those in the wild-type strain [46].

Exogenous Production of Terpenoids via Synthetic Biology Approach (Table 2)

Seco-iridoid is a monoterpenoid indole alkaloid (MIA) with anticancer and insect-repellent activities. The last four steps of its biosynthetic pathway were discovered and reconstituted in another plant, enabling heterologous production of the complex MIA strictosidine [47]. Geranic acid is an attractive monoterpenoid in flavor industry. Introduction of the mevalonate pathway from *Myxococcus xanthus* resulted in production of 193 mg/l of geranic acid in *Pseudomonas putida*, a host with superior tolerance towards geranic acid [48]. Limonene is a monoterpene frequently used in the cosmetic industry. A computational tool, principal component analysis of proteomics (PCAP), was conducted to optimize the heterologous expression of nine mevalonate pathway genes in *E. coli*, leading to greater than 40% improvement of limonene production [49].

As an antimalarial drug, artemisinin is a sesquiterpene naturally produced by *A. annua*, of which the field cultivation yield is low and weather-dependent. A supply increase route was determined via synthetic approaches, which involves metabolic engineering of microbes for the production of its precursor, artemisinic acid, at high titer, productivity and yield, and subsequent chemical conversion to artemisinin [50]. Earlier attempts of artemisinic acid production were made in *E. coli* [15,51,52]. Owing to the poor expression of eukaryotic CYP450s in *E. coli*, later investigations of artemisinic acid production were switched to yeast chassis cells. Expression of CYP71AV1 and its cognate reductase CPR1 from *A. annua*, followed by improvements in the fermentation process, led to production of 40 g/l amorphadiene in *S. cerevisiae*, but an accompanying issue was a dramatic decrease of cell viability [53]. Two reasons may account for the reduction of cell viability. One is phosphate limitation in the feed process, which might direct carbon flux to produce and limit the cell growth. Another reason is the poor coupling between CYP450 and its cognate reductase, which may lead to the generation of reactive oxygen species and a decrease in cell viability [14,50,53]. To solve this problem, a cytochrome *b5*, aldehyde dehydrogenase *ALDH1*, and a putative alcohol dehydrogenase *ADH1* from *A. annua*, were further introduced. Coupled with fermentation process optimization, the engineered yeast strain was able to produce artemisinic acid as highly as at 25 g/l [14], which is key to its successful commercialization by Amyris (Emeryville, CA, USA).

As a diterpenoid representative, tanshinones found in the Chinese medicinal herb *Salvia miltiorrhiza* (also known as danshen in Chinese) exhibit multifunctional bioactivities, such as antioxidant, antitumor, and antibacterial activities [54]. Owing to the lack of information on later steps of synthesis of tanshinones, a synthetic biology approach for tanshinone production was focused on biosynthesis of its precursor, miltiradiene, in *S. cerevisiae*. To enhance metabolic flux channeling to miltiradiene biosynthesis, the modular pathway engineering (MOPE) strategy was applied by using fusion proteins. The fusion of diterpene synthases SmCPS and SmKSL, fusion of GGPP synthase BTS1 and farnesyl diphosphate synthase ERG20, together with tHMGR overexpression, resulted in miltiradiene level of 365 mg/l in yeast diploid strain YJ2X [55]. Furthermore, overexpression of *tHMGR*, *UPC2.1*, a fusion gene of BTS1 and ERG20, as well as a *Sulfolobus acidocaldarius GGPPS*, led to production of 488 mg/l miltiradiene in *S. cerevisiae* during fed-batch fermentation [56]. In addition to miltiradiene biosynthesis, a *S. miltiorrhiza* CYP76AH1 was identified as miltiradiene oxidase, enabling production of the later precursor of tanshinone, ferruginol. Introduction of *CYP76AH1* and the phyto-CYP reductase gene resulted in ferruginol production at 10.5 mg/l in the engineered *S. cerevisiae* strain [57].

Ginsenosides, including pentacyclic type and tetracyclic type, are triterpenoids that have been proven to be the main effective components of the herbal medicine ginseng with antiviral, antitumor, and cholesterol-decreasing activities. The pentacyclic ginsenosides are derived from

β-amyrin. Incorporation of *Glycyrrhiza glabra* β-amyrin synthase, tHMGR, *S. cerevisiae* squalene synthase, and squalene epoxidase led to 107 mg/l of β-amyrin production in the engineered yeast strain [58]. For tetracyclic ginsenoside production, protopanaxadiol is a representative. Introduction of *Panax ginseng* dammarenediol II synthase and codon-optimized protopanaxadiol synthase, along with tHMGR, a CYP450 reductase from *Arabidopsis thaliana*, farnesyl diphosphate synthase, squalene synthase, and 2,3-oxidosqualene synthase, resulted in 1189 mg/l production of protopanaxadiol in *S. cerevisiae* [59]. Other examples are production of ginsenoside compound K (CK), Rh2, and Rg3. A UDP-glycosyltransferase (UGT) UGTPg1 from *P. ginseng* has been proven to convert protopanaxadiol into CK. Overexpression of *UGTPg1*, as well as *tHMGR* and *UPC2.1*, resulted in 1.4 mg/l CK production [60]. Later on, another two UGTs, UGT 45 and UGT 29, from *P. ginseng* were further identified, and the introduction of them into yeast enabled the chassis cell to directly produce Rh2 and Rg3 [61].

The sweetener glycyrrhizin is a bioactive triterpenoid from *Glycyrrhiza*, with huge economic value. CYP88D6 was identified as the β-amyrin 11-oxidase in the glycyrrhizin biosynthetic pathway. Coexpression of CYP88D6 and β-amyrin synthase resulted in production of 11-oxo-β-amyrin, a possible important intermediate of glycyrrhizin biosynthesis, in yeast [62]. Saikosaponins are triterpenoids originally accumulated in *Bupleurum falcatum* and exhibit multiple bioactivities. Recently, a CYP450 gene from *B. falcatum*, named as *CYP716Y1*, was reported to be involved in the oxidation of saikosaponins. Similar to a previous approach, combined expression of *CYP716Y1* with additional genes from other plant species allows production of monoglycosylated saponins in yeast. Further, a cyclodextrin-based culturing strategy was developed to sequester triterpenes for improving their productivity [63].

Carotenoids are tetraterpenoid representatives and have been widely applied in the food, chemical, and healthcare industries. A β-carotene hydroxylase gene from algae was incorporated into a designed carotenoid biosynthetic pathway, which exhibited higher carotenoid production in yeast cells [64]. In addition to exploration of more effective catalytic elements for carotenoid synthesis, many efforts have been taken to fine-tuning the heterologous pathway to improve their final production. For example, controllable assembly and expression of the β-carotene biosynthetic pathway can be achieved by adopting *GAL10–GAL1* bidirectional promoters [65]. In another study, sequential control mediated by environmental glucose concentration was used to modulate expression of pathway genes and utilization of intermediates, resulting in 1156 mg/l production of carotenoid [66].

Concluding Remarks and Future Perspectives

Higher fungi have been used as medicinal and edible materials for thousands of years and have the ability to produce considerable amounts of bioactive secondary metabolites. In recent years, they have also attracted extensive interest in the production of useful terpenoids. As a result of evolution, higher fungi are inherently tolerant and have suitable expression systems for the production of their native terpenoids, which was still mysterious and was regarded as its unique merit as cell factory for terpenoid biosynthesis. However, studies on the production of terpenoids by higher fungi are far from sufficient either in depth or in extent, which may be attributed to (i) limited knowledge on metabolism and regulation of higher fungi, and (ii) their corresponding immature genetic manipulation systems.

Understanding biosynthetic pathways and their complicated regulation is critical to comprehend the determinants for improving terpenoid biosynthesis. For example, in the case of GAs, some definite steps were identified for formation of the terpenoid skeletons; however, the enzymes responsible for postmodification of these skeletons, the majority of which are believed to be CYP450s, are still unknown (Figure 2). Recently, more and more 'omics' information of higher fungi is being released [22,67–71], and such studies including genomics and transcriptomics may lead to not only the clarification of terpenoid biosynthetic steps, their biosynthetic

Outstanding Questions

The current common problems in studying the higher-fungus secondary metabolism are: (i) the biosynthetic pathway of interested secondary metabolites is unclear, thus construction of the heterologous biosynthetic pathway of GAs in other hosts is not easy; (ii) the regulatory mechanism of secondary metabolite (including terpenoid) biosynthesis is not well understood; (iii) genetic modification of higher fungi is still not so easy as the gene transformation system is immature, and further development of genetic manipulation tools such as application and modification of the CRISPR-Cas system is critical.

With trackable genetic backgrounds, shorter fermentation cycle, and mature zymotechnics, heterologous hosts have multiple merits as cell factories for terpenoid biosynthesis. However, their ability to achieve economical production metrics will likely be a big challenge.

mechanisms, and physiological significance but also further development of genetic tools, including discovery of more determinates for improving HR efficiency, promoters with different expression strength, and so forth.

Compared with the achievement of genetic engineering of the cell factories of *E. coli* and yeasts, studies on higher fungi are obviously lagging behind. Manipulations are still limited to single gene modification or individual pathways. Notably, gene deletion was only available in a very few species of higher fungi and their corresponding efficiency was still very low. Such problems may be solved by developing genome editing tools such as CRISPR-Cas [72–74] and group II introns [75] for different species of higher fungi, which accordingly enable combinatorial manipulations and result in extensive impact on production of useful secondary metabolites.

Synthetic biology approaches support identification of the terpenoid biosynthetic pathway and have also been widely applied to heterologous production of terpenoids. With trackable genetic

Table 2. Exogenous Production of Terpenoids via a Synthetic Biology Approach

Terpenoids	Host	Synthetic Biology Approach	Titer or Yield
Monoterpenoids or Their Precursors			
Strictosidine	*Nicotiana benthamiana*	Expression of eight genes encoding the (seco)iridoid biosynthetic pathway, two genes for precursor formation and two genes for downstream alkaloid biosynthesis	Not mentioned [47]
Geranic acid	*P. putida*	Introduction of the mevalonate pathway from *Myxococcus xanthus*	193 mg/l [48]
Limonene	*E. coli*	Optimize the heterologous expression of nine mevalonate pathway genes by PCAP	605 mg/l [49]
Precursors of Sesquiterpenoids			
Artemisinic acid	*S. cerevisiae*	Expression of CYP71AV1 and its cognate reductase CPR1 from *A. annua*, a cytochrome *b5*, aldehyde dehydrogenase ALDH1, and a putative alcohol dehydrogenase ADH1 from *A. annua*	25 g/l [14]
Precursors of Diterpenoids			
Miltiradiene	Diploid yeast strain YJ2X	MOPE strategy	365 mg/l [55]
	S. cerevisiae	Overexpression of tHMGR, UPC2.1, a fusion gene of BTS1 and ERG20, as well as a *Sulfolobus acidocaldarius* GGPPS	488 mg/l [56]
Ferruginol	*S. cerevisiae*	Introduction of CYP76AH1 from *S. miltiorrhiza* and phyto-CYP reductase	10.5 mg/l [57]

Table 2. (continued)

Terpenoids	Host	Synthetic Biology Approach	Titer or Yield
Triterpenoids or Their Precursors			
β-Amyrin	*S. cerevisiae*	Incorporation of *Glycyrrhiza glabra* β-amyrin synthase, tHMGR, *S. cerevisiae* squalene synthase, and squalene epoxidase	107 mg/l [58]
Protopanaxadiol	*S. cerevisiae*	Introduction of *Panax ginseng* dammarenediol II synthase and codon-optimized protopanaxadiol synthase, along with tHMGR, a cytochrome P450 reductase from *Arabidopsis thaliana*, farnesyl diphosphate synthase, squalene synthase, and 2,3-oxidosqualene synthase	1189 mg/l [59]
Ginsenoside compound K (CK)	*S. cerevisiae*	Overexpression of UGTPg1 from *P. ginseng*, as well as tHMGR and UPC2.1	1.4 mg/l [60]
Rh2	*S. cerevisiae*	Introduction of UGTPg45	1.45 μmol/g DCW [61]
Rg3	*S. cerevisiae*	Introduction of UGTPg45 and UGTPg21	3.49 μmol/g DCW [61]
11-Oxo-β-amyrin	*S. cerevisiae*	Coexpression of CYP88D6 from *Glycyrrhiza* and β-amyrin synthase	Not mentioned [62]
Saponins	*S. cerevisiae*	Expression of *CYP716Y1* from *B. falcatum* and additional genes from other plant species	Not mentioned [63]
Tetraterpenoids			
β-Carotene	*Kluyveromyces marxianus*	Incorporated a β-carotene hydroxylase gene from algae into a designed carotenoid biosynthetic pathway	244.7 μg/g DCW [64]
	S. cerevisiae	Controllable assembly and expression of the heterologous β-carotene biosynthetic pathway by adopting *GAL10–GAL1* bidirectional promoters	7.41 mg/g DCW [65]
Carotenoid	*S. cerevisiae*	Sequential control mediated by environmental glucose concentration was used to modulate expression of heterologous pathway genes and utilization of the intermediates	1156 mg/l (20.79 mg/g DCW) [66]

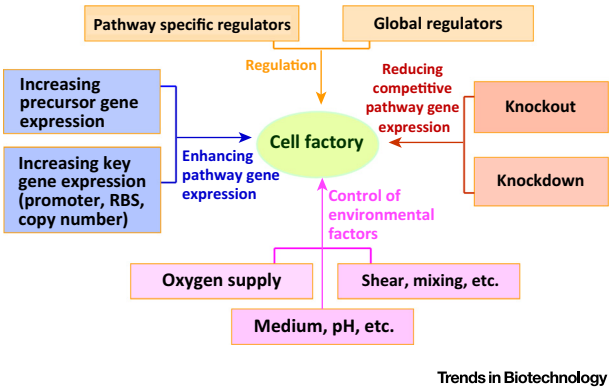

Figure 3. Strategies Empowering Smart Higher-Fungus Cell Factory Construction (Adapted from [2]). Abbreviation: RBS, ribosomal binding site.

Box 2. Strategies Empowering Smart Higher-Fungus Cell Factory Construction

With the advances in metabolic engineering, synthetic biology, and process engineering, combinatorial strategies, including enhancing pathway gene expression, reducing competitive pathway gene expression, controlling regulation and environmental factors, allow construction of higher-fungus cell factories for target terpenoid production (Figure 3). For optimizing pathway gene expression, increasing precursor supply, improving the expression of key genes via changing the promoter strength, ribosomal binding site (RBS), and copy numbers, and reducing competitive pathway via knockout or knockdown of the branched genes are some of the commonly adopted approaches. By contrast, different levels of regulation play nonignorable roles in terpenoid synthesis by native higher-fungi hosts. For example, spore accumulation was always observed in cells with increased GA production (our unpublished data). With the aid of omics research, more and more candidates related to either pathway specific regulation or global regulation will emerge and will be characterized. Employing their function for improving terpenoid synthesis in higher fungi will be the further step. In addition, responding to different environmental factors such as oxygen supply, medium, pH, shearing, mixing, and so on, various cellular physiological and metabolic responses could be obtained. Therefore, control of environmental factors may provide a useful means for improving terpenoid fermentation, especially in large-scale cultivations.

backgrounds, shorter fermentation cycle, and mature zymotechnics, heterologous hosts have multiple merits as cell factories for terpenoid biosynthesis. However, their ability to achieve economical production metrics will likely be a big challenge. From up-to-date reports, we only found artemisinic acid production as one successful commercial case, which only requires a simple and highly efficient photochemical conversion to its final product artemisinin (Table 2). Potential product toxicity may be the common bottleneck, because many bioactive terpenoids generally cause cell toxicity (Table 1) and these products are new to the heterologous hosts. Specific transporters and regulation mechanisms, which are responsible to pump out these toxic products and alleviate product toxicity, are often absent in the heterologous hosts. To address this limitation, strategies could be learned from nature, for example, introducing novel transporters to pump out toxic products, which also requires the exploitation of information from multilevel 'omics' investigation on higher fungi.

By integrating genetic manipulation platforms and 'omics' research, more progress will be made to uncover the mechanisms of terpenoid biosynthesis by higher fungi. As a result, a better ability of engineering will be obtained with regard to commercialization of target compounds, especially on construction of smart higher-fungus cell factories for terpenoid biosynthesis (Figure 3, Box 2).

Acknowledgments

H.X. gratefully acknowledges the Scientific Research Foundation for the Returned Overseas Chinese Scholars of the Ministry of Education of China and the Open Funding Project of the State Key Laboratory of Bioreactor Engineering of ECUST for financial support. J-J.Z. thanks the National Natural Science Foundation of China (no. 21176153) and National 973 program (no. 2012CB721006).

References

1. Zhang, L. (2015) Secondary metabolites and bioactivities from higher fungi in China. *Mini Rev. Med. Chem.* 15, 157–177
2. Qin, H. *et al.* (2016) Cell factories of higher fungi for useful metabolite production. *Adv. Biochem. Eng. Biotechnol.* 155, 199–235
3. Thomas, J.P. *et al.* (2001) Phase I clinical and pharmacokinetic trial of irofulven. *Cancer Chemother. Pharmacol.* 48, 467–472
4. Seiden, M.V. *et al.* (2006) A phase II study of irofulven in women with recurrent and heavily pretreated ovarian cancer. *Gynecol. Oncol.* 101, 55–61
5. Yan, K. *et al.* (2006) Biochemical characterization of the interactions of the novel pleuromutilin derivative retapamulin with bacterial ribosomes. *Antimicrob. Agents Chemother.* 50, 3875–3881
6. Tang, Y.Z. *et al.* (2012) Pleuromutilin and its derivatives – the lead compounds for novel antibiotics. *Mini Rev. Med. Chem.* 12, 53–61
7. Tang, W. *et al.* (2006) Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Life Sci.* 80, 205–211
8. Xu, J.W. *et al.* (2012) Enhancement of ganoderic acid accumulation by overexpression of an N-terminally truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase gene in the basidiomycete *Ganoderma lucidum*. *Appl. Environ. Microbiol.* 78, 7968–7976
9. Xu, J.W. *et al.* (2010) Production of individual ganoderic acids and expression of biosynthetic genes in liquid static and shaking cultures of *Ganoderma lucidum*. *Appl. Microbiol. Biotechnol.* 85, 941–948
10. Zhang, Y.H.P. and Huang, W.D. (2012) Constructing the electricity–carbohydrate–hydrogen cycle for a sustainability revolution. *Trends Biotechnol.* 30, 301–306
11. Zhu, Z. *et al.* (2014) A high-energy-density sugar biobattery via a synthetic enzymatic pathway. *Nat. Commun.* 5, 3026
12. Zhao, H. (2013) *Synthetic Biology: Tools and Applications,* Academic Press
13. Yu, J.L. *et al.* (2014) Direct biosynthesis of adipic acid from a synthetic pathway in recombinant *Escherichia coli*. *Biotechnol. Bioeng.* 111, 2580–2586
14. Paddon, C.J. *et al.* (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 496, 528–532
15. Tsuruta, H. *et al.* (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin in *Escherichia coli*. *PLoS ONE* 4, e4489
16. Schmidt-Dannert, C. (2015) Biosynthesis of terpenoid natural products in fungi. *Adv. Biochem. Eng. Biotechnol.* 148, 19–61
17. Wawrzyn, G.T. *et al.* (2012) Discovery and characterization of terpenoid biosynthetic pathways of fungi. *Methods Enzymol.* 515, 83–105
18. Kelner, M.J. *et al.* (1987) Preclinical evaluation of illudins as anticancer agents. *Cancer Res.* 47, 3186–3189
19. Wawrzyn, G.T. *et al.* (2012) Draft genome of *Omphalotus olearius* provides a predictive framework for sesquiterpenoid natural product biosynthesis in basidiomycota. *Chem. Biol.* 19, 772–783
20. Shang, R.F. *et al.* (2014) Synthesis and biological evaluation of new pleuromutilin derivatives as antibacterial agents. *Molecules* 19, 19050–19065
21. Shiao, M.S. (1992) Triterpenoid natural-products in the fungus *Ganoderma lucidum*. *J. Chin. Chem. Soc.* 39, 669–674
22. Chen, S. *et al.* (2012) Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. *Nat. Commun.* 3, 913
23. Li, G. *et al.* (2006) A highly efficient polyethylene glycol-mediated transformation method for mushrooms. *FEMS Microbiol. Lett.* 256, 203–208
24. Li, S. *et al.* (2001) Efficient transformation of the medicinal mushroom *Ganoderma lucidum*. *Plant Mol. Biol. Rep.* 19, 383
25. Sun, L. *et al.* (2001) PEG-mediated transformation of *Lentinus edodes*. *Acta Bot. Sin.* 43, 1089–1092
26. Kuo, C.Y. and Huang, C.T. (2008) A reliable transformation method and heterologous expression of beta-glucuronidase in *Lentinula edodes*. *J. Microbiol. Methods* 72, 111–115
27. Chai, R. *et al.* (2013) Beta-glucan synthase gene overexpression and beta-glucans overproduction in *Pleurotus ostreatus* using promoter swapping. *PLoS ONE* 8, e61693
28. Lin, Y.J. *et al.* (2013) Enhancement of heterologous gene expression in *Flammulina velutipes* using polycistronic vectors containing a viral 2A cleavage sequence. *PLoS ONE* 8, e59099
29. Lugones, L.G. *et al.* (2004) The SC15 protein of *Schizophyllum commune* mediates formation of aerial hyphae and attachment in the absence of the SC3 hydrophobin. *Mol. Microbiol.* 53, 707–716
30. Ohm, R.A. *et al.* (2010) An efficient gene deletion procedure for the mushroom-forming basidiomycete *Schizophyllum commune*. *World J. Microbiol. Biotechnol.* 26, 1919–1923
31. De Jong, J.F. *et al.* (2010) Inactivation of ku80 in the mushroom-forming fungus *Schizophyllum commune* increases the relative incidence of homologous recombination. *FEMS Microbiol. Lett.* 310, 91–95
32. Nakazawa, T. *et al.* (2011) Efficient gene targeting in delta Cc.ku70 or delta Cc.lig4 mutants of the Agaricomycetes *Coprinopsis cinerea*. *Fungal Genet. Biol.* 48, 939–946
33. Godio, R.P. *et al.* (2007) A squalene epoxidase is involved in biosynthesis of both the antitumor compound clavaric acid and sterols in the basidiomycete *H. sublateritium*. *Chem. Biol.* 14, 1334–1346
34. Papa, I.A. *et al.* (2006) Increasing pleuromutilin activity of *Clitopilus passeckerianus* by chemical mutagenesis and improvement of production medium. *Philipp. Agric. Sci.* 89, 20–33
35. Benkortbi, O. *et al.* (2007) Batch kinetics and modelling of pleuromutilin production by *Pleurotus mutilis*. *Biochem. Eng. J.* 36, 14–18
36. Zou, X. *et al.* (2012) Fed-batch fermentation for pleuromutilin production by *Pleurotus mutilis*. *Res. J. Biotechnol.* 7, 67–72
37. Bailey, A.M. *et al.* (2013) *Methods of increasing yields of pleuromutilins,* Glaxo Group Limited & the University of Bristol, (United Kingdom) US 20130017608 A1
38. Wu, G.S. *et al.* (2013) Anti-cancer properties of triterpenoids isolated from *Ganoderma lucidum* – a review. *Exp. Opin. Investig. Drugs* 22, 981–992
39. Fang, Q.H. and Zhong, J.J. (2002) Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. *Biotechnol. Prog.* 18, 51–54
40. Tang, Y.J. *et al.* (2009) Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and ganoderma polysaccharides by medicinal mushroom *Ganoderma lucidum*. *Bioresour. Technol.* 100, 1852–1859
41. Liang, C.X. *et al.* (2010) Enhanced biosynthetic gene expressions and production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under phenobarbital induction. *Appl. Microbiol. Biotechnol.* 86, 1367–1374
42. Xu, Y.N. *et al.* (2013) Induced effect of Na^+ on ganoderic acid biosynthesis in static liquid culture of *Ganoderma lucidum* via calcineurin signal transduction. *Biotechnol. Bioeng.* 110, 1913–1923
43. Xu, Y.N. *et al.* (2014) Induction of ganoderic acid biosynthesis by Mn^{2+} in static liquid cultivation of *Ganoderma lucidum*. *Biotechnol. Bioeng.* 111, 2358–2365
44. Xu, Y.N. and Zhong, J.J. (2012) Impacts of calcium signal transduction on the fermentation production of antitumor ganoderic acids by medicinal mushroom *Ganoderma lucidum*. *Biotechnol. Adv.* 30, 1301–1308
45. Zhang, W. and Tang, Y.J. (2008) A novel three-stage light irradiation strategy in the submerged fermentation of medicinal mushroom *Ganoderma lucidum* for the efficient production of ganoderic acid and ganoderma polysaccharides. *Biotechnol. Prog.* 24, 1249–1261
46. Zhou, J.S. *et al.* (2014) Enhanced accumulation of individual ganoderic acids in a submerged culture of *Ganoderma lucidum* by the overexpression of squalene synthase gene. *Biochem. Eng. J.* 90, 178–183
47. Miettinen, K. *et al.* (2014) The seco-iridoid pathway from *Catharanthus roseus*. *Nat. Commun.* 5, 3606
48. Mi, J. *et al.* (2014) De novo production of the monoterpenoid geranic acid by metabolically engineered *Pseudomonas putida*. *Microb. Cell Fact.* 13, 170

49. Alonso-Gutierrez, J. *et al.* (2015) Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. *Metab. Eng.* 28, 123–133
50. Paddon, C.J. and Keasling, J.D. (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nat. Rev. Microbiol.* 12, 355–367
51. Hedl, M. *et al.* (2004) Class II 3-hydroxy-3-methylglutaryl coenzyme A reductases. *J. Bacteriol.* 186, 1927–1932
52. Martin, V.J.J. *et al.* (2001) The in vivo synthesis of plant sesquiterpenes by *Escherichia coli*. *Biotechnol. Bioeng.* 75, 497–503
53. Westfall, P.J. *et al.* (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* 109, E111–E118
54. Zhou, L.M. *et al.* (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol.* 45, 1345–1359
55. Zhou, Y.J. *et al.* (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. *J. Am. Chem. Soc.* 134, 3234–3241
56. Dai, Z. *et al.* (2012) Production of miltiradiene by metabolically engineered *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 109, 2845–2853
57. Guo, J. *et al.* (2013) CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12108–12113
58. Dai, Z.B. *et al.* (2014) Producing aglycons of ginsenosides in bakers' yeast. *Sci. Rep.* 4, 3698
59. Dai, Z. *et al.* (2013) Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. *Metab. Eng.* 20, 146–156
60. Yan, X. *et al.* (2014) Production of bioactive ginsenoside compound K in metabolically engineered yeast. *Cell Res.* 24, 770–773
61. Wang, P. *et al.* (2015) Production of bioactive ginsenosides Rh2 and Rg3 by metabolically engineered yeasts. *Metab. Eng.* 29, 97–105
62. Seki, H. *et al.* (2008) Licorice beta-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin. *Proc. Natl. Acad. Sci. U.S.A.* 105, 14204–14209
63. Moses, T. *et al.* (2014) Combinatorial biosynthesis of sapogenins and saponins in *Saccharomyces cerevisiae* using a C-16 alpha hydroxylase from *Bupleurum falcatum*. *Proc. Natl. Acad. Sci. U.S.A.* 111, 1634–1639
64. Chang, J.J. *et al.* (2015) Integrating an algal beta-carotene hydroxylase gene into a designed carotenoid-biosynthesis pathway increases carotenoid production in yeast. *Bioresour. Technol.* 184, 2–8
65. Xie, W.P. *et al.* (2014) Construction of a controllable beta-carotene biosynthetic pathway by decentralized assembly strategy in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 111, 125–133
66. Xie, W.P. *et al.* (2015) Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae*. *Metab. Eng.* 28, 8–18
67. Bao, D. *et al.* (2013) Sequencing and comparative analysis of the straw mushroom (*Volvariella volvacea*) genome. *PLoS ONE* 8, e58294
68. Morin, E. *et al.* (2012) Genome sequence of the button mushroom *Agaricus bisporus* reveals mechanisms governing adaptation to a humic-rich ecological niche. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17501–17506
69. Ohm, R.A. *et al.* (2010) Genome sequence of the model mushroom *Schizophyllum commune*. *Nat. Biotechnol.* 28, 957–963
70. Yap, H.Y.Y. *et al.* (2014) The genome of the tiger milk mushroom Lignosus rhinocerotis, provides insights into the genetic basis of its medicinal properties. *BMC Genomics* 15, 635
71. Zhu, Y.J. *et al.* (2015) Chromosome-level genome map provides insights into diverse defense mechanisms in the medicinal fungus *Ganoderma sinense*. *Sci. Rep.* 5, 11087
72. Bao, Z.H. *et al.* (2015) Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. *ACS Synth. Biol.* 4, 585–594
73. Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823
74. Zetsche, B. *et al.* (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771
75. Enyeart, P.J. *et al.* (2014) Biotechnological applications of mobile group II introns and their reverse transcriptases: gene targeting RNA-seq, and non-coding RNA analysis. *Mob. DNA* 5, 2
76. Jaspers, N.G. *et al.* (2002) Anti-tumour compounds Illudin S and Irofulven induce DNA lesions ignored by global repair and exclusively processed by transcription- and replication-coupled repair pathways. *DNA Repair (Amst.)* 1, 1027–1038
77. Chen, H.P. *et al.* (2014) Four new sesquiterpenoids from fruiting bodies of the fungus *Inonotus rickii*. *J. Asian Nat. Prod. Res.* 16, 581–586
78. Ma, K. *et al.* (2014) New benzoate derivatives and hirsutane type sesquiterpenoids with antimicrobial activity and cytotoxicity from the solid-state fermented rice by the medicinal mushroom *Stereum hirsutum*. *Food Chem.* 143, 239–245
79. Ishikawa, N.K. *et al.* (2001) Antimicrobial cuparene-type sesquiterpenes, enokipodins C and D, from a mycelial culture of *Flammulina velutipes*. *J. Nat. Prod.* 64, 932–934
80. Wang, Y.Q. *et al.* (2012) Bioactive sesquiterpenoids from the solid culture of the edible mushroom *Flammulina velutipes* growing on cooked rice. *Food Chem.* 132, 1346–1353
81. Kanokmedhakul, S. *et al.* (2012) Cytotoxic sesquiterpenes from luminescent mushroom *Neonothopanus nambi*. *Tetrahedron* 68, 8261–8266
82. Hartley, A.J. *et al.* (2009) Investigating pleuromutilin-producing *Clitopilus* species and related basidiomycetes. *FEMS Microbiol. Lett.* 297, 24–30
83. Shi, X.W. *et al.* (2012) Secoscabronine M, a novel diterpenoid from the Chinese bitter mushroom *Sarcodon scabrosus*. *Chirality* 24, 386–390
84. Bai, R. *et al.* (2015) Striatoids A–F, cyathane diterpenoids with neurotrophic activity from cultures of the fungus *Cyathus striatus*. *J. Nat. Prod.* 78, 783–788
85. Han, J. *et al.* (2013) Anti-inflammatory and cytotoxic cyathane diterpenoids from the medicinal fungus *Cyathus africanus*. *Fitoterapia* 84, 22–31
86. Hirota, M. *et al.* (2002) Anti-inflammatory compounds from the bitter mushroom Sarcodon scabrosus. *Biosci. Biotechnol. Biochem.* 66, 179–184
87. Xu, J.W. *et al.* (2010) Biotechnological production and application of ganoderic acids. *Appl. Microbiol. Biotechnol.* 87, 457–466
88. Weng, C.J. *et al.* (2007) The anti-invasive effect of lucidenic acids isolated from a new *Ganoderma lucidum* strain. *Mol. Nutr. Food Res.* 51, 1472–1477
89. Kim, K.H. *et al.* (2013) Lanostane triterpenoids from the mushroom *Naematoloma fasciculare*. *J. Nat. Prod.* 76, 845–851
90. Ma, K. *et al.* (2014) Ganoboninketals A–C, antiplasmodial 3,4-seco-27-norlanostane triterpenes from *Ganoderma boninense* Pat. *J. Nat. Prod.* 77, 1847–1852
91. Hien, B.T. *et al.* (2013) Cattienoids A–C, three novel steroids from the mushroom *Tomophagus cattienensis*. *Fitoterapia* 91, 125–127