Insight into yeast: A study model of lipid metabolism and terpenoid biosynthesis

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Abstract

With the development of transcriptomics, metabolomics, proteomics, and mathematical modeling, yeast *Saccharomyces cerevisiae* is recently considered as a model studying strain by biologists who try to reveal the mystery of microorganic metabolism or develop heterologous pharmaceutical and economic products. Among *S. cerevisiae* metabolic research, lipid metabolism always attracts great interest because of its dominant role in cell physiology. Related researchers have developed multiple functions from cell membrane component such as adjustment to changing environment and impact on protein folding. Nowadays, many common human diseases such as diabetes mellitus, Alzheimer's disease, obesity, and atherosclerosis are related to lipid metabolism, which makes the study of lipids a desperate need. In addition to lipid metabolism, the study of the native mevalonic acid (MVA) pathway in *S. cerevisiae* has increased exponentially because of its huge potential to produce economically important products terpenoids. With the progress of technology in gene engineering and metabolic engineering, more and more biosynthetic pathways will be developed and put into industrial application. Volume 00, Number 00, Pages 1–6, 2014

Keywords: *biosynthesis, lipids, metabolism, Saccharomyces cerevisiae, terpenoids*

Abbreviations: *BTS1, GGPP synthase; DMAPP, dimethylallyl diphosphate; Erg1, oxido squalene synthase; Erg2, sterol C-8 isomerase; Erg4, sterol C-24 reductase; Erg6, sterol C-24 methyltransferase; Erg7, lanosterol synthase; Erg8, phosphomevalonate kinase; Erg9, squalene synthase; Erg10, acetyl-CoA-C-acetyltransferase; Erg12, mevalonate kinase; Erg19, mevalonate diphosphate decarboxylase; Erg20, geranyl/farnesyl diphosphate synthase; Erg25, C-4 sterol methyloxidase; Erg26, C-3 sterol dehydrogenase; Erg27, 3-keto sterol reductase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl pyrophosphat; GPP, geranyl diphosphate; HMG1/HMG2, hydroxymethylglutaryl-CoA reductase 1/2; IPP, isopentenyl diphosphate; MVA, mevalonic acid.*

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1. Introduction

Lipids are usually divided into the following three main classes: sterols, phospholipids, and sphingolipids. By gene deletion [1] and high-throughput screening methods [2], sterol synthesis process in *Saccharomyces cerevisiae* has been clearly explained, involving all the genes and enzymes in upstream mevalonic acid (MVA) pathway and downstream ergosterol synthesis pathway. Phospholipids are synthesized using the following three pathways: cytidine diphosphate (CDP)–diacylglycerol (DAG) pathway, Kennedy pathway [3], and exogenous lysolipid metabolism (ELM) pathway [4]. However, sphingolipid synthesis pathway in *S. cerevisiae* remains unknown.

Lipids have great pharmaceutical and economical values; for example, ergosterol plays the role of a precursor of vitamin D2 and cortisone [5], and also demonstrates potential in cancer therapy [6]. On the other hand, the upstream MVA pathway and downstream ergosterol synthesis pathway in *S. cerevisiae* harbor huge capacities for manufacturing terpenoids. For example, artemisinic acid [7] and taxadiene [8], which are precursors of artemisinin and taxol, respectively, have already been successfully manufactured using *S. cerevisiae*, and this discovery is regarded as a milestone in synthetic biology. In

conclusion, researches on lipid metabolism and terpenoid biosynthesis have shown great importance, and applications of them will benefit human beings. This review focuses on recent and important progress in these two aspects with *S. cerevisiae* as a studying model.

2. Lipid Metabolism

2.1. Introduction of lipids

Lipids are key components of eukaryotic cells. For their great impact on cell physiology, researches on lipid metabolism have attracted the attention of many biologists. Eukaryotic cells synthesize lipids in multiple pathways to keep the cell in a physically and chemically stable environment [9]. In general, the composition and concentration of lipids are maintained in a constant level, which is usually called lipid homeostasis. Because of the development of transcriptomics, metabolomics, and proteomics, combined with modern detection technology such as gas chromatography, high-efficiency liquid chromatography, and mass chromatography, qualitative and quantitative analyses of lipids at cell, tissue, and organ levels are available [10, 11]. At the same time, researches on lipids have evolved from merely cell membrane components to the metabolism and homeostasis of lipids.

2.2. Lipid metabolism and homeostasis

As a eukaryotic cell, *S. cerevisiae* encodes different genes to manipulate lipids metabolic activities [12]. But only encoded genes can not precisely predict the status of some proteins and enzymes, so researchers also try to discover the details of lipid metabolism and homeostasis in other ways.

First, when exploring a metabolic pathway of lipids, researchers combined gene deletion and high-throughput screening methods to observe the effect of certain genes' deletion. For example, by the systematic analysis of *S. cerevisiae* with some defects, genes responsible for transferring sphingolipids and sterols to cell surface were found; by exploring auxotrophic phenotype, genes responsible for lipids droplet formation were figured out [13, 14]. By gene deletion and high-through screening methods, biologists have identified key genes in sterols synthesis pathway and depicted a clear gene manipulating system of sterol synthesis. But if nonessential genes were knocked out, there would be no obvious phenotype changes. In addition, the synergistic and complementary relationships among genes can also interrupt the result.

Second, when studying the lipids homeostasis of *S. cerevisiae*, researchers found that yeast harbored a complex and precise manipulating system to maintain the constant concentration and composition of lipids. They used thin-layer chromatography, high-efficiency liquid chromatography, liquid mass chromatography, and gas mass chromatography to detect lipid metabolites in cells at a certain time, performed qualitative and quantitative analyses, and speculated the possible metabolic pathways of lipids. Jonikas et al. [15] found that by the deletion of several important genes in phospholipids synthetic CDP–DAG pathway and Kennedy pathway, an unfolded protein response (UPR) would be activated. This UPR detected misfolded proteins in the endoplasmic reticulum and started the transcription and translation of corresponding genes that facilitated proper protein folding [16]. In addition to this, the UPR also functioned to upregulate some key genes responsible for phospholipids synthesis. UPR was thus regarded as an important response toward lipids homeostasis in *S. cerevisiae* [17, 18].

Last but not least, different kinds of lipids have interactive influences and they are not synthesized or degraded in separated ways. In fact, different pathways often share same intermediates or metabolic substrates [19]. So, we should have a whole concept of lipids homeostasis because a small turbulence in one lipid pathway may cause changes in another lipid pathway, and further study is needed to know about these influences. On the other hand, the changes in the concentration and composition of lipids may have an impact on microenvironment of proteins, thus affect protein folding and enzyme bioactivity, so we also need to consider lipids homeostasis in a lipid–protein interactive way [20]. However, up to now, research on the impact of lipids on protein folding has just developed, and more information is needed to explain lipid–protein phenomena.

The importance of lipids to cell physiology has pushed lipids research forward and researchers have made progress in multiple aspects, but to date, some questions such as the process by which a cell adjusts its lipid composition to adapt to adverse environment conditions and how lipids provide a suitable environment for protein activities remain unclear. Obviously, more efforts are needed to answer these questions.

Researchers found that a number of human diseases such as diabetes mellitus, Alzheimer's disease, obesity, and atherosclerosis are connected with lipids, which demonstrated a significant socioeconomic value of lipid study and encouraged a deeper exploration [21, 22].

3. Natural Synthesis of Lipids

3.1. Natural synthesis of sterols

Sterols have great impact on cell membrane, so *S. cerevisiae* harbors complex and precise ways to manipulate sterol synthesis in different phases. In the transcription phase, *S. cerevisiae* has two binuclear cluster transcription factors, Upc2 and Ecm22, to regulate the synthesis of ergosterol [23]. When ergosterol is uptaken by *S. cerevisiae*, the DNA-binding area of Upc2 and Ecm22 binds conserved sequences of promoter, in charge of ergosterol synthesis, which activates the transcription of ergosterol synthesis genes [24]. But in this transcription process, who acts as the ergosterol sensor and whether proteolysis is needed to facilitate transcription are still unknown. In the translation phase, *S. cerevisiae* owns two homologous enzymes Hmg1 and Hmg2, which cooperate as key isoenzymes to regulate the transformation of HMG-CoA to mevalonate. Either Hmg1 or Hmg2 is sufficient for cell growth, so we take Hmg2 as an example. *S. cerevisiae* uses two lipid sensors oxysterol

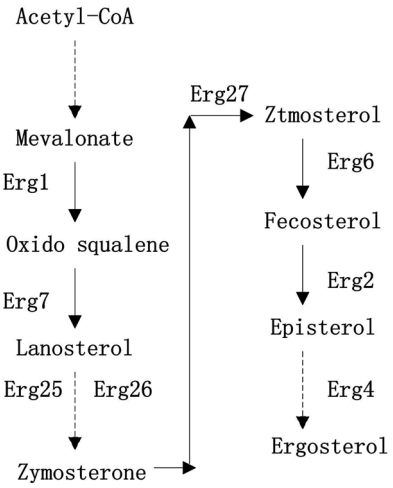

FIG. 1 *Key intermediates and genes in the MVA pathway and sterol synthesis pathway. Solid arrows indicate one-step reactions, whereas dashed arrows denote multistep reactions.*

and isoprenoid geranyl geranyl diphosphate (GGDP) to regulate the degrading rate of Hmg2. When the concentration of GGDP increases, the conformation of Hmg2 changes, which speeds up the Hmg2 degrading rate, finally decreasing the sterol synthesis rate [25, 26]. Up to now, every gene and enzyme in sterol synthesis has been clearly described. Figure 1 shows some important intermediates and genes in the synthesis pathway of sterol.

3.2. Natural synthesis of phospholipids

Yeast has hundreds of phospholipids, located in the whole cell membrane. So, the synthesis of phospholipids also undergoes complex and precise regulation including nutrition identification, lipid–protein cooperation, and transcription control [27]. In the process of phospholipids synthesis, phosphatidic acid (PA) plays an important role. PA is a key intermediate of phospholipids metabolism and synthetic precursor of many phospholipids. By cooperating with transcription repressor Opi1 protein (Opi1), PA regulates the synthesis of major phospholipids, and Opi1 manipulates the translation of many genes in charge of phospholipids metabolism [28]. Recent research shows that this lipid–protein cooperation between PA and Opi1 is induced by pH. When intercellular pH changes, an alteration of electronic properties of lipids follows a series of bioreactions, then further motivated to start phospholipids synthesis [29]. Although this kind of phospholipids synthesis with PA as an intermediate has been discovered, some details remain unknown and more synthetic pathways need to be discovered. Figure 2 shows the chemical structure of PA, the precursor of phospholipids.

Until very recently, the natural synthesis of sphingolipids is still largely vague. Some biologists suggested that PA might play a role in sphingolipids synthesis, but this hypothesis needs more data and experiments to specify the regulation details.

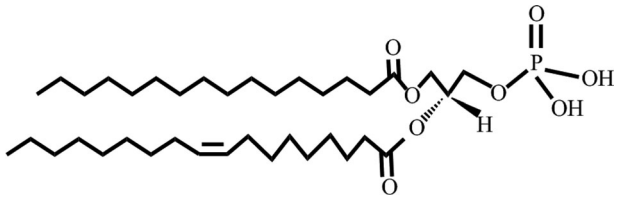

FIG. 2 *Chemical structure of phospholipids precursor PA.*

4. Biosynthesis of Terpenoids

4.1. Introduction to terpenoids

Terpenoids consist of a large and diverse class of natural products that share some common molecular structures. So far, almost all terpenoids are derived from plants. These terpenoids are primary metabolites that regulate plant growth or secondary metabolites, which play an important role in adapting the changing environment. Terpenoids have huge socioeconomic values, and they play roles in many areas such as medicine, fragrances, flavors, and health-promoting agents. For example, natural rubber is a high-yield terpenoids product with annual production around 1×10^7 tons, and is widely used in tires, vibration dampers, and latex products. Even though there are many different kinds of terpenoids, they are similar in structure. All terpenoids are derived from the basic C_5H_8 unit; terpenoids with two C_5H_8 units are called monoterpenoids such as geraniol and menthol, diterpenoids such as phytol and vitamin A have four C_5H_8 units, and triterpenoids consist of six C_5H_8 units and many therapeutic products such as squlenes, ginsenosides belong to this category.

When terpenoids synthesis is put into industrial production, there are some problems. First, as primary and secondary metabolites, terpenoids accumulate in a small amount in plants, which cannot meet the industrial need. Second, some terpenoids are synthesized in certain tissues during certain period, and the synthesis can be easily influenced by environmental conditions such as light and humidity, which makes the production unpredictable. Also, because of the variety of terpenoids, extraction becomes difficult because a single plant can have hundreds of terpenoids and different distributions of these terpenoids sometimes make the issue more complicated [40].

Facing such problems in terpenoids production, biologists took several methods to cope with them, including (1) gene engineering of plants to improve their terpenoids production [41], (2) separate cultivation of tissues such as cell and root that produced terpenoids, and (3) combining gene engineering and protein engineering and then harvesting terpenoids in heterologous microbial hosts. But plant-derived products are often subject to climate disasters, plant diseases, and geographical issues, whereas cell or tissue cultivation sometimes cannot meet the production demands [42]. The third method will be discussed in the following section.

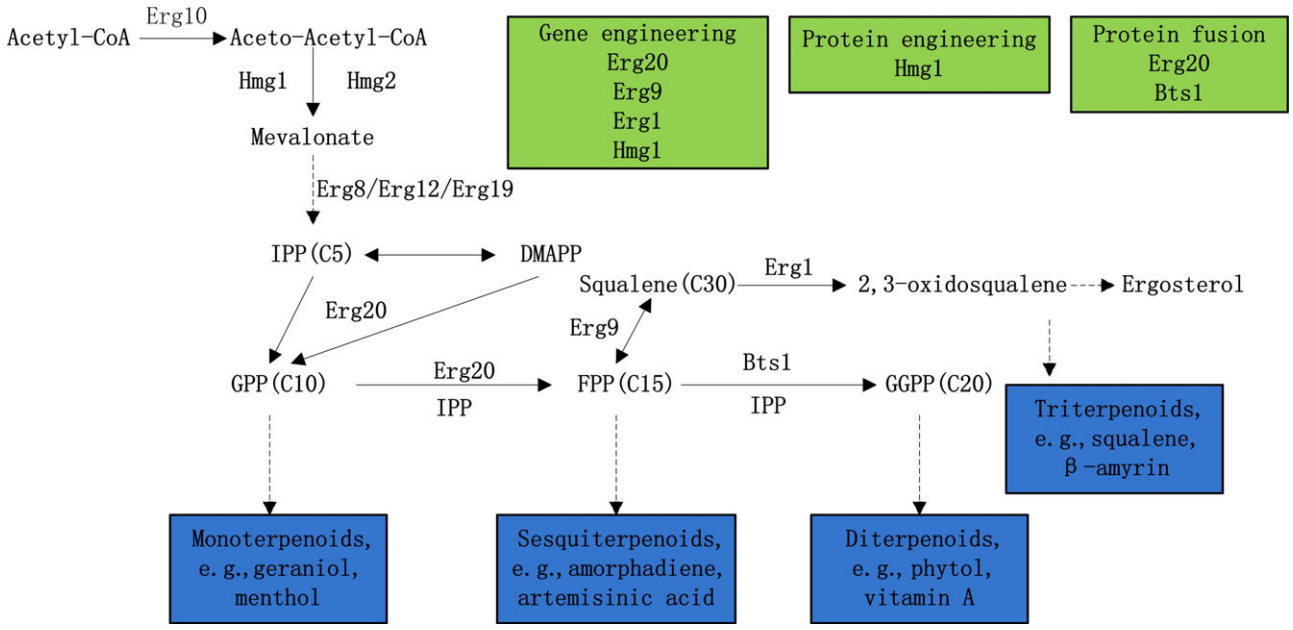

FIG. 3 *Methods of gene engineering, protein engineering, and protein fusion for producing interesting terpenoids. Solid arrows indicate one-step reactions, whereas dashed arrows denote multistep reactions in yeast. IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl pyrophosphat.*

4.2. Engineering synthesis of terpenoids

Compared with plants, microbial hosts have their unique advantages such as short growth cycle, excellent resistant ability to environment changes, simple culture medium ingredients, and easy for scale-up, finally saving the cost and time to a large extent. In addition, the synthesis of terpenoids is an environmental friendly living system, so there is no toxic emission or utilization of harsh condition.

S. cerevisiae harbors the endogenous MVA pathway and sterol synthesis pathway. There are several genetic methods such as promoter modification, gene knockout, gene mutation, and heterologous gene expression for *S. cerevisiae* to produce terpenoids. Figure 3 shows the MVA pathway, heterologous terpenoids synthesis pathway, and also some key enzymes. From Fig. 3, we see that *S. cerevisiae* has the ability to produce different kinds of terpenoids, including industrial material geraniol, healthcare agent vitamin A, and antimalaria medicine precursor amorphadiene. But according to different target products, different gene regulations are applied. For example, when sesquiterpenoids are target products, researchers usually downregulates Erg9 to decrease the metabolic flow from farnesyl pyrophosphate to squalene but on the contrary when triterpenoids are target products, they upregulates Erg9 to ensure enough triterpenoids precursors.

When producing terpenoids in *S. cerevisiae* through gene engineering, some tools are needed to modify microbial hosts. For example, stable chassis, suitable gene vectors, efficient promoters, and CAD analyzing models are all powerful tools that help in constructing yeasts through engineering synthesis for target products [43].

Up to now, in the trials of synthesizing terpenoids in *S. cerevisiae*, Keasling and co-workers succeeded in producing the antimalarial drug precursor artemisinic acid. This trial was regarded as a milestone in gene engineering, which started the epoch of synthetic biology. They modified *S. cerevisiae* in the following three steps: (1) engineered the farnesyl pyrophosphate (FPP) synthetic pathway to upregulate FPP; at the same time, reduced the metabolic flow to sterol synthesis pathway; (2) introduced amorphadiene synthase gene that transformed FPP to amorphadiene; and (3) introduced a cytochrome P450 that performed a three-step oxidation of amorphadiene; finally, got target product artemisinic acid. Through these genetic modifications, the production of artemisinic acid reached 100 mg/L. Another outstanding synthetic biological case was manufacturing of the taxol precursor taxadiene in *S. cerevisiae*. Jennewein and co-workers [44] (1) upregulated FPP production and downregulated *erg9* gene, (2) integrated a short version of *HMG-CoA* gene, and (3) cloned a taxadiene synthase gene; finally, the taxadiene production reached 8.7 mg/L.

Besides gene engineering, another randomly used terpenoids producing method is protein engineering. Protein engineering is aimed to increase the product yield, create enzymes with higher specificity, and develop new catalytic enzymes [45]. Squalene is a common triterpenoid product and meanwhile important metabolic intermediate of *S. cerevisiae*. Donald et al. [46] applied a truncated Hmg1 that included only the linker and catalytic domain, successfully achieving a 10 times enhancement of squalene production. As for higher specificity of enzymes, Keasling and

TABLE 1 *Engineering synthesis of terpenoids*

Target terpenoid	*Genetic modification*	*Reference*
Patchoulol	Downregulation of Erg9, overexpression of tHmg1	[38, 39]
β-Amyrin	Overexpression of tHmg1, downregulation of Erg7	[32]
Protopanaxadiol	Overexpression of tHmg1, Erg1, Erg9, and Erg20	[33]
Fecosterol	Overexpression of tHmg1 and Are2	[31]
Dammarenediol	Introducing of dammarenediol synthase	[36]
β-Carotene	Overexpression of tHmg1	[34]
Oleanolic acid	Introducing of β-amyrin synthase and CYP716A12	[35]
Linalool	Introducing of Clarkia breweri S-linalool synthase	[30]
Cineole	Overexpression of Hmg2 and Idi1	[51]
β-Bisabolene	Site-saturation mutagenesis A336V, M447H, I562T of γ-humulene synthase	[47]
Longifolene	Site-saturation mutagenesis A317N§, A336S, S484C, I562V of γ-humulene synthase	[47]
α-Longipinene	Site-saturation mutagenesis A336C, T445C, S484C, I562L, M565L of γ-humulene synthase	[47]
Miltiradiene	Fusion of SmCPS and SmKSL, active site engineering of Bts1 and Erg20	[37]

co-workers [47] successfully engineered wild-type γ-humulene synthase from *Abies grandis*. By using site-directed saturation mutagenesis and site-directed mutagenesis, they got several more specific terpene synthases (TPSs) such as sibirene synthase (F321Q, M339A, and M447F) and E-β-farnesene synthase (W315P), whereas the original γ-humulene synthase catalyzed nearly 50 sesquiterpenes.

In addition to gene engineering and protein engineering, protein fusion also demonstrated itself as a popular and useful method for increasing terpenoids production. Protein fusion was usually used to reduce diffusion of intermediates or degradation caused by rival enzymes, finally avoiding losses of intermediates [48]. For example, fusion of endogenous Erg20 and heterologous TPSs showed higher yields than separate ones [49]. Tanshionones were found rich in Chinese herb *Salvia miltiorrhiza* with a variety of biological activities such as an antibacterial and anticancer effect. Miltiradiene was the key precursor of tanshionones. By fusion of Bts1 and Erg20, SmKSL and SmCPS, together with overexpression of tHmg1, Zhou and co-workers improved the titeration of miltiradiene to 365 mg/L in a 15-L bioreactor. The miltiradiene production of original strain which only introduced SmKSL, and SmCPS was almost undetectable. Cases of gene engineering, protein engineering, and protein fusion are also shown in Fig. 3.

Up to now, some successful protein engineering cases are still operated in *Escherichia coli*. For example, Prather and co-workers used double-mutant S239C/G295D and binding residue selection to obtain a 10-fold production improvement in levopimaradiene. We hope that more experiments can be conducted in *S. cerevisiae*, achieving a host chassis development from prokaryotes to eukaryotes. As for protein fusion, a fusion sequence should be carefully arranged because the three-dimensional structure of two closed proteins would be influenced by their expression sequence. For example, in Zongbao's case mentioned above, different expression sequences of SmKSL–SmCPS and SmCPS–SmKSL caused obvious different miltiradiene titer. Some other cases for terpenoids producing ginsenosides and β-amyrin are concluded in Table 1 where, all methods of gene engineering, protein engineering, and protein fusion are mentioned.

4.3. Exploration of terpenoids synthesis: The road ahead

In recent decades, the development of theoretical knowledge and application technology in synthetic biology gives us new insights into plants and microorganisms, making the microbial terpenoids synthesis no longer a dream. Also, microbial terpenoids synthesis has gradually demonstrated itself many advantages over plant terpenoids synthesis. Microbial terpenoids synthesis always takes place in safe, well-known, and industrial microbes that have tractable genetic pathways, so developing more model microorganisms is desperately needed. Also, continuing development of new chassis, expression vectors, and creation of novel enzymes that catalyze unnatural reactions will help to expand the range of products in modern engineered microorganisms.

We believe that when more resources and information can be utilized, microbial terpenoids synthesis will become as powerful and flexible as synthetic chemistry, bringing new products with additional values [50].

5. Conclusions

As eukaryotic microorganism, *S. cerevisiae* has some beneficial characters such as simple ingredients of the medium, convenient genetic modification, simple genome, and conservative metabolic pathways. It is thus always in the research focus of many biologists and regarded as a model study strain. As lipids have great impact on physiological and chemical status of *S. cerevisiae*, lipid metabolism and homeostasis have emerged as promising researches in metabolic engineering. Even though some pathways and genes have been found, lipids adaptive mechanism to the changing environment including pressure sense, gene inducement, and protein expression remains unknown. On the other hand, *S. cerevisiae* harbors the MVA pathway and sterol synthesis pathway, providing great opportunities for terpenoids engineering synthesis. Many products have been manufactured and applied in therapy, fragrance, and flavor areas, but we still need to further combine theoretical knowledge and practical technology in synthetic biology to create more valuable products, benefiting society and people.

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