CHAPTER TEN

Engineering Microbes to Synthesize Plant Isoprenoids

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Abstract

Humans constantly look for faster, more economical, and more sustainable ways to produce chemicals that originally harvested from nature. Over the past two decades, substantial progress has been made toward this goal by harnessing enzymes and cells as biocatalysts. For example, enzymes of slow-growing plants can be reconstituted in microbes, which empower them with the ability to produce useful plant metabolic compounds from sugars faster than plants. In this chapter, we provide protocols for producing isoprenoids – a large group of useful natural products – in microbes. It has been found that expression of genes encoding plant enzymes and selected endogenous genes must be delicately adjusted in microbes, otherwise isoprenoid production is negatively affected. Therefore, we focus on how to balance gene expression in *Escherichia coli* and use process engineering to increase its isoprenoid production. We also introduce our recent work on the use of microbial consortia and provide protocols for using yeast to help *E. coli* functionalize its isoprenoid product. Together, the methods and protocols provided here should be useful to researchers who aim to use microbes to synthesize novel isoprenoids.

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1. INTRODUCTION

Isoprenoids are a large group of natural products, which contain tens of thousands of chemicals with diverse functional groups (Cuellar & van der Wielen, 2015; Krivoruchko & Nielsen, 2015). Presently, many isoprenoids can be found on the global market as pharmaceuticals, nutrients, fragrances, flavoring substances, bulk chemicals, and fuels (Ajikumar et al., 2008). Most of these compounds were originally discovered in plants and – for many of them – extraction of plant materials remains a major production route (Ajikumar et al., 2010). However, it becomes increasingly difficult to meet the growing demand of many isoprenoids from plant extraction, due to the slow growth rate and low isoprenoid content of plants. For some relatively simple isoprenoids such as carotenoids, total chemical synthesis methods have been developed (Shen et al., 2011), but it is challenging to similarly produce more complex compounds, especially those with cyclic structures and multiple oxygenated functional groups. To tackle this challenge, different approaches are under investigation, including genetic modification of plants to increase isoprenoid content (Wilson & Roberts, 2014), development of plant cell culture in which plant biomass accumulates faster (Wilson, Cummings, & Roberts, 2014), and heterologous production by using engineered microbes (Biggs, De Paepe, Santos, De Mey, & Kumaran Ajikumar, 2014). We discuss the last approach in this chapter.

Microbes are ideal hosts for producing isoprenoids, because they grow fast, require little land/water resources, and naturally produce the building blocks of all isoprenoids (Fig. 1), isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). There are also abundant genetic tools available for engineering microbes. Theoretically, any plant-produced isoprenoid can be produced in microbes – using its endogenous IPP/DMAPP as substrates – by expressing plant isoprenoid synthases (IsoSs) and functionalizing enzymes (Fig. 1).

Intensive research has been done to harness the biosynthetic capacity of bacteria for isoprenoid production. In bacteria, glycolytic intermediates are converted into IPP and DMAPP via methylerythritol pyrophosphate (MEP) pathway (Fig. 1), which consumes energy (in terms of ATP) and reducing equivalents (in terms of NADPH and NADH). MEP pathway flux is weak in nonengineered bacteria due to their low cellular demand for isoprenoids, so various strategies have been developed to increase flux of the pathway (Fig. 2), including overexpression of the rate-limiting enzymes

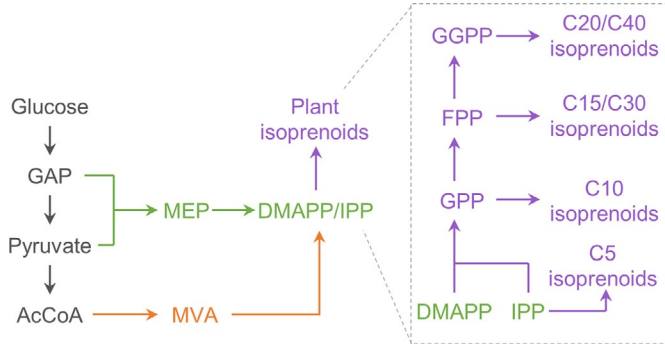

Fig. 1 Schematic illustration of metabolic pathways for production of plant isoprenoids in microbes. Both MEP and MVA pathways can convert intermediates of central metabolism into IPP and DMAPP, two building blocks for all isoprenoids. IPP and DMAPP can be converted into various plant isoprenoids by plant isoprenoid synthases and functionalizing enzymes. *GAP*, glyceraldehyde 3-phosphate; *AcCoA*, acetyl CoA; *MEP*, methylerythritol 4-phosphate; *MVA*, mevalonate; *DMAPP*, dimethylallyl pyrophosphate; *IPP,* isopentenyl pyrophosphate; *GPP*, geranyl pyrophosphate; *FPP*, farnesyl pyrophosphate; *GGPP*, geranylgeranyl pyrophosphate.

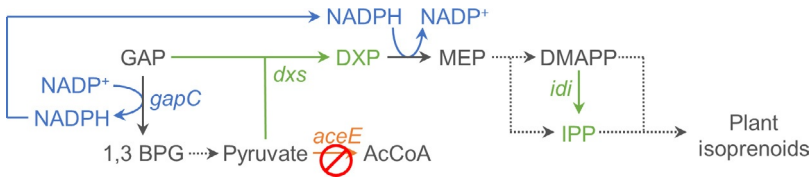

Fig. 2 Three rational strategies to increase flux through the MEP pathway – overexpression of rate-limiting genes (*dxs* and *idi*), inactivation of competing pathway (△*aceE*), and enrichment of essential cofactors (NADPH). *GAP*, glyceraldehyde 3-phosphate; *1,3 BPG,* 1,3-bisphosphoglyceric acid; *DXP*, 1-deoxy-D-xylulose 5-phosphate; *MEP*, methylerythritol 4-phosphate; *DMAPP*, dimethylallyl pyrophosphate; *IPP*, isopentenyl pyrophosphate; *Dxs*, the gene encoding 1-deoxy-D-xylulose 5-phosphate synthase; *aceE*, the gene encoding subunit of E1p component of pyruvate dehydrogenase complex; *idi*, isopentenyl diphosphate isomerase.

(Yuan, Rouviere, Larossa, & Suh, 2006), inactivation of competing pathways by gene knockout (Alper, Jin, Moxley, & Stephanopoulos, 2005), enrichment of the cofactors by engineering central metabolism (Martínez, Zhu, Lin, Bennett, & San, 2008), and combinatorial manipulations coupled with high-throughput screening (Alper & Stephanopoulos, 2007; Tyo, Ajikumar, & Stephanopoulos, 2009). Among them, the first strategy was direct and effective – expressing DXS (the first enzyme in the pathway) pushed carbon flux from central metabolism into the pathway, and having

more IDI (the last enzyme in the pathway) efficiently isomerized DMAPP into IPP, which is needed in larger quantity than DMAPP in synthesis of most (C10 and heavier) isoprenoids (Yuan et al., 2006). However, one complication associated with this strategy is that there is an optimal expression status for the MEP enzymes: when their levels are too low the overall pathway flux is limited; when expression levels were too high, isoprenoid production could be inhibited, possibly due to accumulation and active efflux of an MEP intermediate (MEC), as revealed in a metabolite profiling study (Zhou, Zou, Stephanopoulos, & Too, 2012a, 2012b). This optimal expression status is also heavily influenced by downstream IsoSs so it must be experimentally determined each time when new IsoSs are expressed or IsoSs' expression is altered (Ajikumar et al., 2010).

In 2010, we developed a method that can rapidly locate optimal expression level of both MEP enzymes and downstream IsoSs (Ajikumar et al., 2010) and have applied the method to improve production of taxadiene, the scaffold molecule of anticancer drug paclitaxel (a C20 isoprenoid). According to this method, important MEP enzymes are lumped together and regarded as a module, and downstream IsoSs (geranylgeranyl pyrophosphate synthase and taxadiene synthase in the case) are treated as another module. Expression level of each module can be systematically altered by using various promoters and replication origins, and a large number of combinations can be subsequently generated by combining variants of each module. Quantifying isoprenoid production for all combinations of modular gene expression then reveals the optimal expression levels for both modules. In Section 2 of this chapter, we will describe detailed protocols for implementing a simplified version of this method for producing C20 isoprenoid scaffold molecules (ISMs). In the following sections, we will provide detailed protocols for scaling up isoprenoid production in laboratory bioreactor and oxygenating C20 ISMs by using microbial consortia. We conclude with a discussion of how to adapt protocols described in this chapter for producing isoprenoids other than the C20 ones.

2. IMPROVING PRODUCTION OF C20 ISMs BY MODULAR GENETIC ENGINEERING OF *ESCHERICHIA COLI*

E. coli is a model Gram-negative bacterium that has been widely used for both academic study and industrial application within the biotech industry. There are many techniques that can alter activity of an enzyme in *E. coli* – they can be classified into two groups, those altering quantity of

the enzyme and those affecting specific enzymatic activity. The latter group includes protein engineering (eg, mutagenesis, domain shuffling, fusion, and truncation), expression of chaperones that help folding of the target enzyme, and altering local concentration of its substrates (Zhou et al., 2012a, 2012b). The former group primarily focuses on altering the copy number of the gene that encodes the enzyme, the promoter that drives transcription of the gene, and the ribosomal binding site that influences translation efficiency of the gene's transcript (Ajikumar et al., 2010). Because the former group is less dependent on the sequence and function of the target enzyme, they have been widely used to genetically engineer *E. coli*. Here, we illustrate how to locate optimal expression levels of MEP genes and IsoSs for isoprenoid production by using different promoters and replication origins.

Our prior study on taxadiene production indicated that MEP genes need to be expressed at relatively low level – while IsoSs should be strongly over-expressed (Ajikumar et al., 2010). This study allows simplification of the procedure for locating optimal gene expression level (Fig. 3): MEP genes are all integrated into genome of *E. coli*, under three different promoters (P_{trc}, P_{T5}, and P_{T7}); IsoSs are expressed on three multicopy plasmids (p5, p10, and p20), all with a strong promoter (P_{T7}). Each multicopy plasmid is transformed into an engineered *E. coli*, creating nine strains in total. Analyzing their isoprenoid production could reveal the optimal expression levels of both MEP genes and IsoSs.

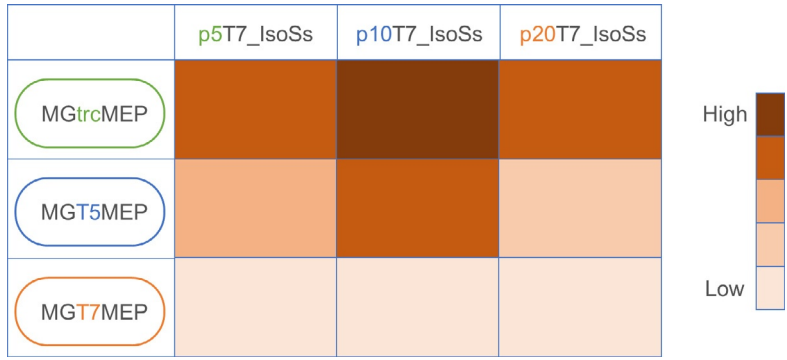

Fig. 3 Illustration of locating optimal expression levels of MEP genes and isoprenoid synthases by modular genetic engineering of *E. coli*. Color indicates level of isoprenoid production. *MGtrcMEP*, *E. coli* MG1655 (Δ*recA*, Δ*endA*, DE3, Δ*araA*::P_{trc}-*dxs-idi-ispDF*); *MGT5MEP*, *E. coli* MG1655 (Δ*recA*, Δ*endA*, DE3, Δ*araA*::P_{T5}-*dxs-idi-ispDF*); *MGT7MEP*, *E. coli* MG1655 (Δ*recA*, Δ*endA*, DE3, Δ*araA*::P_{T7}-*dxs-idi-ispDF*).

2.1 Constructing Expression Vectors for IsoSs

2.1.1 IsoS-Encoding Genes

Our focus here is on C20 isoprenoids because their scaffold molecules are easy to handle – they are not volatile, yet still can cross cell membrane, thus simplifying purification. The example used in the following protocols is miltiradiene, the scaffold molecule of tanshinone, a C20 therapeutic isoprenoid found in *Salvia miltiorrhiza* (Zhou, Qiao, Edgar, & Stephanopoulos, 2015). As all C20 ISMs, miltiradiene is derived from geranylgeranyl pyrophosphate (GGPP) (Fig. 1). Cyclization of GGPP into miltiradiene can be catalyzed by a fusion enzyme in *E. coli*, encoded by *ksl_cps*, which can be synthesized through commercial gene synthesis service. We have cloned a GGPP synthase from *Taxus canadensis* into the three expression vectors, which can be used to supply GGPP for production of any C20 isoprenoids (Fig. 4).

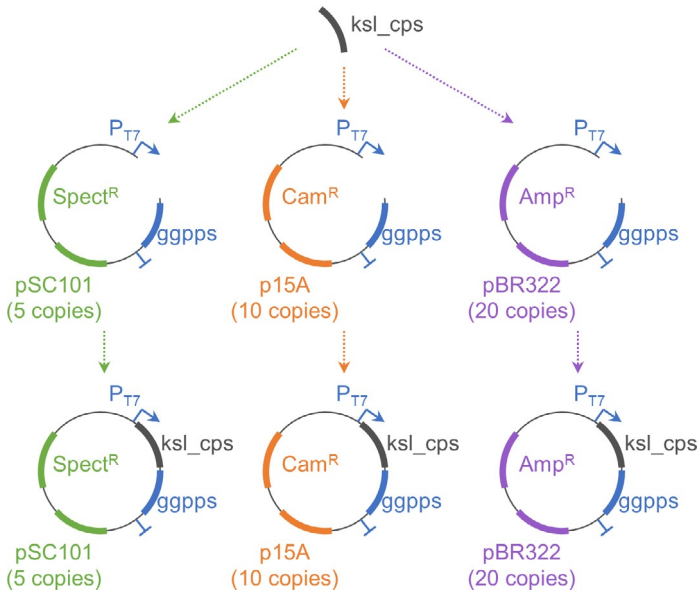

Fig. 4 Construction of expression vectors for C20 isoprenoid synthases by using CLIVA method. *Ksl_cps*, the gene encoding miltiradiene synthase; *Ggpps*, the gene encoding geranylgeranyl pyrophosphate synthase; P_{T7}, T7 promoter; $Spect^R$, spectinomycin resistance gene; Cam^R, chloramphenicol resistance gene; Amp^R, ampicillin resistance gene; *pSC101*, pSC101 replication origin, whose copy number is *E. coli* is estimated to be 5; *p15A*, p15A replication origin, whose copy number in *E. coli* is estimated to be 10; *pBR322*, pBR322 replication origin, whose copy number in *E. coli* is estimated to be 20.

2.1.2 Primer Design

Ksl_cps is inserted into the three vectors by using CLIVA method (Zou, Zhou, Stephanopoulos, & Too, 2013). DNA fragments used in CLIVA method need to be generated by using PCR, where modified oligos are used to introduce phosphorothioate bonds to DNA fragments (Fig. 5). The phosphorothioate bonds can be cleaved by using iodine and ethanol at basic condition, generating long, specific sticky ends, which ensure accurate annealing of the DNA fragments. The fragments are covalently linked after the annealed fragments are transformed into *E. coli* (Fig. 5). A common forward primer is designed for amplifying all three plasmids – it is pXT7_F with sequence "GATCCAAT*CCAGGTCT*AA," where "*" denotes a phosphorothioate bond – and the common reverse primer for all three plasmids is pXT7-R with sequence "CATCATGC*CCATGGTA*TA." Forward primer for *ksl_cps* (Sequence 1) is ksl_cps_F with sequence "TACCATGG*GCATGATG*AGCCTGGCATTTAAT," and reverse primer for ksl_cps is ksl_cps_R with sequence "AGACCTGG*ATTGGATC*TTAGGCAACCGGCTC."

2.1.3 PCR Reaction

For each PCR reaction, the following components are mixed in a PCR tube: 25 μL of Q5 master mixture (NEB), 2.5 μL of forward primer (10 μ*M*), 2.5 μL of reverse primer (10 μ*M*), 5 μL of plasmid or IsoSC gene (1 ng/μL), and 20 μL of ultrapure water. The capped PCR tube is incubated in a thermocycler according to the following program: 98°C for 30 s, 35 cycles of (98°C for 10 s, 55°C for 30 s, and 72°C for *X* s), 72°C for

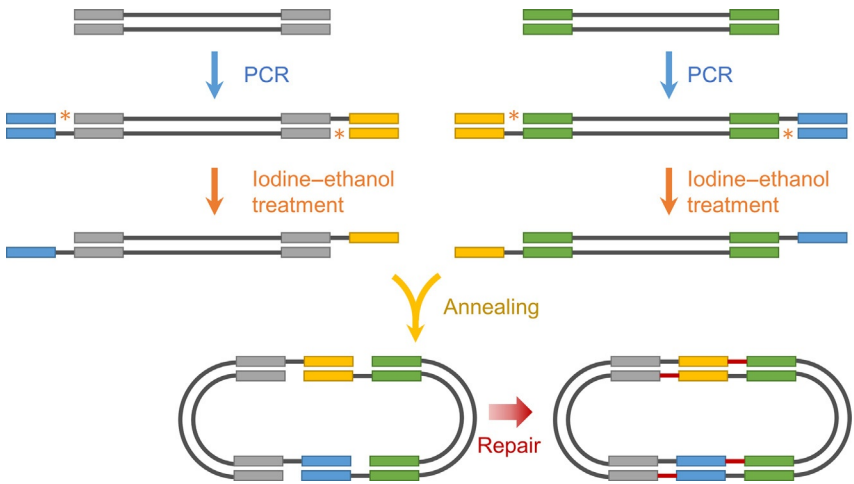

Fig. 5 Schematic illustration of the CLIVA method.

SEQUENCE 1 Sequence of *ksl_cps*

ATGAGCCTGGCATTTAATCCGGCAGCAACCGCATTTAGCGGTAATGGTGCACGTAGC
CGTCGTGAAAACTTTCCGGTTAAACATGTTACCGTTCGTGGTTTTCCGATGATTACCA
ATAAAAGCAGCTTTGCCGTTAAATGCAATCTGACCACCACCGATCTGATGGGCAAAA
TTGCAGAAAAATTCAAAGGCGAGGATAGCAATTTTCCGGCAGCCGCAGCAGTTCAGC
CTGCAGCAGATATGCCGAGCAATCTGTGTATTATTGATACCCTGCAGCGTCTGGGTG
TTGATCGTTATTTTCGTAGCGAAATTGATACCATCCTGGAAGATACCTATCGTCTGTGG
CAGCGTAAAGAACGTGCAATTTTTAGCGATACCGCAATTCATGCAATGGCATTTCGTC
TGCTGCGTGTTAAAGGTTATGAAGTTAGCAGCGAAGAACTGGCACCGTATGCAGATC
AAGAACATGTGGATCTGCAGACCATTGAAGTTGCAACCGTTATTGAACTGTATCGTGC
AGCACAAGAACGTACCGGTGAAGATGAAAGCAGCCTGAAAAAACTGCATGCATGGA
CCACCACATTTCTGAAACAGAAACTGCTGACCAATAGCATCCCGGATAAAAAACTGC
ACAAACTGGTGGAATACTATCTGAAAAACTTTCACGGCATTCTGGATCGTATGGGTGT
TCGTCAGAATCTGGATCTGTACGATATTAGTTATTACCGTACCAGCAAAGCAGCCAAT
CGTTTTAGTAATCTGTGCTCCGAAGATTTTCTGGCATTTGCACGTCAGGATTTTAACAT
TTGTCAGGCACAGCATCAGAAAGAACTGCAGCAGCTGCAACGTTGGTATGCCGATTG
TAAACTGGATACCCTGAAATATGGTCGTGATGTTGTTCGTGTTGCAAATTTTCTGACCA
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CTGACCCTGGATGATTATCTGAGCGCAAGCTGGGTTAGCATTGGTTGTCGTATTTGTA
TTCTGATGTCCATGCAGTTCATTGGCATCAAACTGTCAGATGAAATGCTGCTGAGCGA
AGAATGTATTGATCTGTGTCGTCATGTTAGCATGGTGGATCGCCTGCTGAATGATGTT
CAGACCTTTGAAAAAGAACGCAAAGAGAATACCGGTAATAGCGTTACCCTGCTGCTG
GCAGCAAATAAAGATGATAGCAGTTTTACCGAAGAAGAGGCAATTCGTATTGCAAAA
GAAATGGCCGAATGTAATCGTCGTCAGCTGATGCAGATTGTGTATAAAACCGGTACA
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GTATGCAAGCGGTGATGAATTTACCAGTCCGCAGCAGATGATGGAAGATATGAAAAG
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GGTGGTGGTTCTGCAAGCCTGAGCAGCACCATTCTGAGCCGTAGTCCGGCAGCACGT
CGTCGTATTACACCGGCAAGCGCAAAACTGCATCGTCCGGAATGTTTTGCAACCAGC
GCATGGATGGGTAGCAGCAGCAAAAATCTGAGCCTGAGCTATCAGCTGAACCACAA
AAAAATCAGCGTTGCAACCGTTGATGCACCGCAGGTTCATGATCACGATGGCACCAC
CGTTCATCAGGGTCATGATGCAGTTAAAAACATTGAAGATCCGATCGAATATATCCGT
ACCCTGCTGCGTACCACCGGTGATGGTCGTATTAGCGTTAGCCCGTATGATACCGCA
TGGGTTGCAATGATTAAAGATGTTGAAGGTCGTGATGGTCCGCAGTTTCCGAGCAGC
CTGGAATGGATTGTTCAGAATCAGCTGGAAGATGGTAGCTGGGGTGATCAGAAACTG
TTTTGTGTTTATGATCGTCTGGTGAATACCATTGCATGTGTTGTTGCACTGCGTAGCTG

SEQUENCE 1 Sequence of *ksl_cps*—cont'd

GAATGTTCATGCACATAAAGTTAAACGTGGCGTGACCTATATCAAAGAAAACGTGGA
TAAACTGATGGAAGGCAACGAAGAACATATGACCTGTGGTTTTGAAGTTGTTTTTCCG
GCACTGCTGCAGAAAGCAAAAAGCCTGGGTATCGAAGATCTGCCGTATGATTCACCG
GCAGTTCAAGAGGTTTATCATGTTCGTGAACAAAAACTGAAACGCATTCCGCTGGAAA
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CAGTACCGCATTTGCATTTATGCAGACCAAAGATGAGAAATGCTATCAGTTCATCAAA
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GCAGTTGCCAAAACCTATTATTACCGTGCATATCATGCAGCCGATACCATTGATACCC
ATATGTTCAAAGTTCTGTTTGAGCCGGTTGCCTAA

120 s, and held at 16°C, where *X* is 20 times length (kb) of the gene or vector (eg, *X* is 50 for a 2.5 kb gene and 140 for a 7 kb vector).

2.1.4 DNA Electrophoresis and Recovery

Ten microliters of 6× DNA loading dye are added to each PCR reaction mixture, which is briefly vortexed and loaded into a well in a 0.8%

agarose-TAE gel that is prestained with SYBR-Safe DNA Gel Stain. The electrophoresis is carried out at 120 V for 40 min, and the DNA bands are carefully excised on Blue Light Transilluminator (pearl biotech). DNA is recovered from the gel by using DNA Gel Extraction Kit by following manufacture's instruction (at the last step of DNA extraction, DNA is eluted with 40 μL of ultrapure water).

2.1.5 Iodine Treatment and Ethanol Precipitation

The following components are mixed in a 1.7-mL Axygen centrifuge tube: 5.5 μL of 1 *M* Tris solution (pH 9), 10 μL of 30 g/L iodine solution (solvent is ethanol), and 40 μL of the purified PCR product from the last step. The solution is incubated at 70°C for 5 min by using a water bath, and standard sodium acetate ethanol precipitation method is used to purify the treated DNA (at the last step, 15 μL of ultrapure water is used to resuspend the DNA).

2.1.6 DNA Assembly

By this point, there are four purified, digested DNA samples – *ksl_cps*, p5T7 fragment, p10T7 fragment, and p20T7 fragment. *Ksl_cps* is assembled with each of the three vector fragments (Fig. 4). In each case, 3 μL of ksl_cps and 3 μL of a vector fragment are mixed in a PCR tube, which is then incubated in a thermocycler according to the following program: 80°C for 60 s, 50°C for 60 s, and gradually decreased to 16°C at the rate of 0.1°C/s.

2.1.7 Cell Transformation

Two microliters of each DNA assembly reaction mixture are added into 50 μL of DH5α electrocompetent *E. coli* (commercially available), which is electroporated in a 0.1-cm cuvette at 1.8 kV. Two hundred microliters of SOC medium are added into the cuvette to dilute the cell suspension, and the diluted solution is transferred into a 14-mL round-bottom Falcon tube, which is incubated at 37°C/250 rpm for 30 min. Two hundred microliters of the cell suspension are plated onto a LB agar plate with proper antibiotics (50 mg/L spectinomycin for vector p5T7-ksl_cps-ggpps, 34 mg/L chloramphenicol for p10T7-ksl_cps-ggpps, and 50 mg/L carbenicillin for vector p20T7-ksl_cps-ggpps), which is incubated at 37°C for 8–24 h. One colony is picked from each plate, and inoculated into 5 mL of LB medium with proper antibiotics in a 50-mL Falcon tube, which is then incubated at 37°C/250 rpm for 8–24 h. Plasmid is extracted from each cell culture by using a commercial Plasmid Miniprep Kit, and verified via Sanger sequencing.

2.2 Testing the Expression Vectors

2.2.1 Competent Cell Preparation and Cell Transformation

Strains MGtrcMEP, MGT5MEP, and MGT7MEP (Fig. 3) are first made into electrocompetent cells: a colony is inoculated into 10 mL of LB medium in 50 mL of Falcon tube, and it is incubated at 37°C/250 rpm until cell density reaches 0.5 (OD600), after which the cell pellet is washed three times by using 10% glycerol and resuspended in 200 μL of 10% glycerol. Each strain is transformed with the three IsoSC-expressing vector individually by using electroporation as described in Section 2.1.7 (1 μL of the plasmid is used in each transformation). One colony of each resulting strain is inoculated into 5 mL of LB medium with proper antibiotics in a 50-mL Falcon tube, which is then incubated at 37°C/250 rpm for 8–24 h. Five hundred microliters of the cell culture are mixed with 500 μL of 80% glycerol solution (solvent is water) to make *E. coli* glycerol stock, which can be stored at −80°C freezer for up to 5 years.

2.2.2 Preparation of E. coli Seed Culture

Small amounts of *E. coli* glycerol stock are inoculated into 1 mL of LB medium with proper antibiotics – held in 14 mL of Falcon tube – by using disposable 1 μL inoculation loop. The tube is incubated at 37°C/250 rpm for 10–16 h.

2.2.3 Preparation of 1000× K3 Trace Elements Stock Solution

The following components are dissolved in 1 L of deionized water: 5 g $CaCl_2{\cdot}2H_2O$, 1.6 g $MnCl_2{\cdot}4H_2O$, 0.38 g $CuCl_2{\cdot}2H_2O$, 0.5 g $CoCl_2{\cdot}6H_2O$, 0.94 g $ZnCl_2$, 0.0311 g H_3BO_3, and 0.4 g $Na_2EDTA{\cdot}2H_2O$. The mixture is autoclaved at 121°C for 15 min.

2.2.4 Preparation of K3 Basal Medium

Four grams of $(NH_4)_2HPO_4$ and 13.3 g of KH_2PO_4 are dissolved in 0.9 L of deionized water, and the solution is autoclaved at 121°C for 15 min.

2.2.5 Preparation of K3 Master mix

Two point five milliliters of 0.1 *M* ferric citrate solution (autoclaved), 1 mL of 4.5 g/L thiamine solution (sterilized by using 0.2 μm filter), 3 mL of 4 m*M* Na_2MoO_4 (autoclaved), 1 mL of 1000 × K3 trace elements stock solution, and 1 mL of 1 *M* $MgSO_4$ solution (autoclaved) are mixed in a sterile 50-mL Falcon tube. The resulting solution can be stored at room temperature for no more than 3 weeks.

2.2.6 Testing C20 Isoprenoid Production in Test Tube

Ninety milliliters of K3 basal medium, 0.17 mL of K3 master mix, 10 mL of 200 g/L xylose solution (autoclaved), and 100 μL of 50% (v/v) Sigma antifoam B (autoclaved) are mixed aseptically in a sterile container. One milliliter of the mixture is transferred to a sterile VWR 16 × 100 mm (diameter and height) round-bottom culture tube, and 10 μL of fully grown *E. coli* seed culture is added. The tube is incubated at 30°C/250 rpm until cell density reaches 0.5–1.0 (OD600), and 1 μL of 100 m*M* IPTG solution is added to induce isoprenoid production. The tube is then incubated at 22°C/250 rpm for 72 h, and 100 μL of the cell culture is withdrawn at the end for isoprenoid analysis.

2.2.7 Extraction of C20 Isoprenoids for GCMS Analysis

One hundred microliters of cell suspension, 100 μL of ethyl acetate, and 100 μL of 0.5 mm (diameter) glass beads are added into a 1.5-mL Eppendorf safe-lock tube. The tube is vortexed at 2000 rpm for 20 min by using VWR heavy-duty vortex, and then centrifuged at 18,000 × *g* for 2 min. Twenty microliters of the ethyl acetate phase are carefully withdrawn (without touching the aqueous phase) for GCMS analysis.

2.2.8 GCMS Analysis

One microliter of the ethyl acetate phase is injected into GCMS injector at 250°C – C20 ISMs can be vaporized at the temperature. Agilent HP-5ms column is generally suitable for analyzing C20 ISMs, but oven temperature rising program needs to be customized to achieve good separation of target compounds in each case. The program for miltiradiene is 100°C for 1 min, ramping up to 175°C at 15°C/min, ramping up to 220°C at 4°C/min, ramping up to 290°C at 50°C/min, and 290°C for 1 min. Helium is used as carrier gas at 1 mL/min. Mass spectrometry is operated at scan mode (40–400 *m/z*). Total ion counts are used for quantification of C20 isoprenoids with calibration curves constructed by using authentic standards.

3. IMPROVING PRODUCTION OF C20 ISMs BY OPTIMIZING BIOREACTOR OPERATION

Once gene expression levels of the MEP pathway and those of IsoSs are optimized, the resulting strain can be cultured in well-controlled bioreactor to further increase the isoprenoid titer. The higher product titer and large volume of bioreactor culture facilitate purification of desired

isoprenoids in laboratory scale. Usually, following bioreactor optimization, milligrams of C20 isoprenoids can easily be obtained, which could be used in NMR analysis to confirm compound structures, or used as substrate to identify downstream functionalizing enzymes in enzymatic reactions. In this section, we provide detailed protocols for improving titer of C20 ISMs via bioreactor optimization.

3.1 Bioreactor Specifications

A lab scale bioreactor (1–10 L) can be used in this experiment and should include (1) a dissolved oxygen (DO) probe and a stirrer that can be controlled according to online DO reading, (2) a pH probe and two pump heads that can be used to add acid and alkaline to maintain pH, (3) a temperature probe and a heating/cooling system, (4) two pump heads that are used to feed xylose solution (carbon source) and diammonium phosphate solution (nitrogen and phosphorus source), (5) a sparger that is connected to compressed air source via a flow rate controller, (6) a chilled condenser that reduces loss of volume via evaporation, (7) a sampling port, and (8) an inoculation port.

3.2 Protocol for Operating the Bioreactor

3.2.1 Preparation of the Bioreactor

One liter bioreactor is used here as an example with working volume of 500 mL. The bioreactor is assembled according to manufacturer's instruction, and 450 mL of K3 basal medium (Section 2.2.4) is added. Its pH probe is calibrated by using pH 7 and pH 4 standard solution before the reactor is autoclaved at 121°C for 15 min, after which if DO probe is a polargraphic one it needs to be polarized by connecting it to DO meter overnight before calibration. DO probe is calibrated by using nitrogen (0) and air (100%).

3.2.2 Initiating Bioreactor Run and Bioreactor Setting

The following components are added aseptically into the bioreactor through the inoculation port: 850 μL of sterile K3 master mix (Section 2.2.5), 50 mL of 200 g/L xylose solution (autoclaved), 0.5 mL of 50% Sigma antifoam B solution (autoclaved), and 5 mL of fully grown seed culture (Section 2.2.2). Agitation speed is set at 280 rpm, temperature is set at 30°C, and air flow is set at 0.5 L/min. pH is controlled at 7 by using 240 g/L NaOH solution and 0.5 *M* HCl solution. Temperature is programmed to drop to 22°C when DO decreases to 40%. When temperature reaches 22°C, 0.5 mL of 100 m*M* IPTG stock solution is added. Agitation speed is increased (up to 800 rpm, this threshold may vary depending on the bioreactor) when

DO drops below 30%. One milliliter of 50% Sigma antifoam B solution (autoclaved) is added in case of foaming.

3.2.3 Feeding the Bioreactor

E. coli needs large quantity of carbon, nitrogen, and phosphorus to achieve high cell density during bioreactor cultivation. These elements are supplied in the form of xylose and diammonium phosphate over the course of fermentation. It is recommended to pump 500 g/L xylose solution (autoclaved) and 400 g/L diammonium phosphate solution (autoclaved) into the bioreactor at a constant rate. Such linear feeding strategy is easy to implement and can be used to control cell growth under carbon-limited condition. Xylose feeding is started when xylose concentration drops below 10 g/L. Optimal rate of xylose feeding needs to be determined empirically, and often lies in the range between 5 and 15 g/day. Feed rate of the diammonium phosphate solution is always set to be 10% of that of the xylose solution. The bioreactor is stopped at 120 h after IPTG induction.

3.2.4 Sampling Bioreactor and HPLC Analysis

Samples are taken every 24 h for cell density (OD600), GCMS, and HPLC analysis. Samples for GCMS analysis are processed as described in Sections 2.2.7 and 2.2.8. One milliliter of cell suspension is taken for HPLC analysis: it is clarified by centrifugation at $16{,}000 \times g$ for 1 min, and the supernatant is sterilized by using 13 mm 0.2 μm Nylon filter. Ten microliters of the filtrate is injected into Aminex HPX-87H column. The mobile phase is 5 m*M* sulfuric acid, column temperature is 50°C, and each run lasts for 20 min. The HPLC analysis reveals concentration of xylose and fermentation by-products such as acetate.

3.2.5 Purification of Isoprenoids

C20 ISMs can be extracted by ethyl acetate and purified by using HPLC with C18 column and acetonitrile as mobile phase. Because this is a standard procedure in organic chemistry, detailed protocols are not described here.

4. OXYGENATING C20 ISMs BY USING *E. COLI–SACCHAROMYCES CEREVISIAE* COCULTURE

The last two steps of the MEP pathway are catalyzed by iron–sulfur-containing proteins, which are hypersensitive to oxidizing agents – they can be inactivated even by molecular oxygen (Artsatbanov et al., 2012).

Yet functionalization of C20 ISMs often requires oxygenation reactions that are catalyzed by cytochrome P450 (CYP), which often generate large amounts of reactive oxygen species (Zhou et al., 2015). As a result, previous attempts revealed that introduction of CYP into *E. coli* disturbed its MEP pathway and substantially reduced isoprenoid titer (Ajikumar et al., 2010). To address this incompatibility, we spatially separated the CYPs for oxygenating C20 ISMs from the upstream pathways by distributing them into two microbes (Fig. 6) – the first microbe produced an isoprenoid scaffold that can diffuse into the second species of the consortium where it is oxygenated by CYPs (Zhou et al., 2015). This way, reactive oxygen species generated by CYPs would not destroy the iron–sulfur clusters that are located in a different cell.

Here we provide detailed protocols for expression of a CYP in *S. cerevisiae*, characterization of the *S. cerevisiae*, and coculture of the *S. cerevisiae* with an *E. coli* that produces isoprenoid scaffold.

4.1 Expression of a CYP in *S. cerevisiae*

4.1.1 Constructing Yeast Expression Vector for CYP

We have constructed integrative expression vector pYPRC15-URA-UGPD, which can be used to express a CYP and a CYP reductase (CPR) in *S. cerevisiae* (Fig. 7). It is based on pUC19 that can be replicated in *E. coli* and contains two 500 base-pair homologous arms that direct integration into YPRC15 locus on yeast genome. The vector also contains a CYC terminator, an enhanced GPD promoter, and a URA3 marker. Forward primer for amplifying this plasmid is pYPRC15_F with sequence "CATCATCA*TCACCATC*AC,", and the reverse primer is

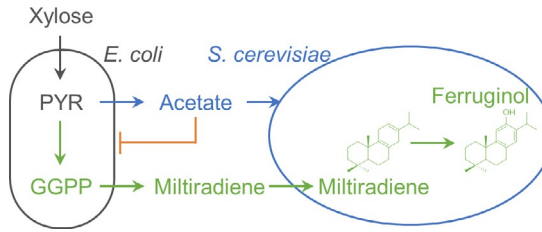

Fig. 6 Oxygenating C20 isoprenoid scaffold molecule by using mutualistic microbial consortium. Miltiradiene is used here as an example – it is produced by *E. coli* and oxidized into ferruginol in *S. cerevisiae*. *PYR*, pyruvate; *GGPP*, geranylgeranyl pyrophosphate; *E. coli* produces acetate as a by-product that inhibits its own growth, which is used here to build a mutualistic relationship between the two species – acetate serves as carbon source of the yeast, which in return removes the inhibitor (acetate) for *E. coli*.

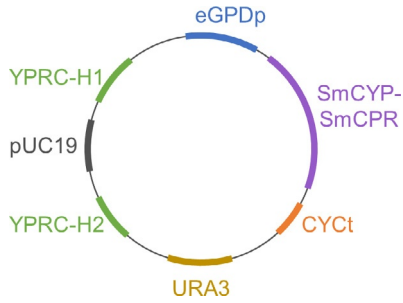

Fig. 7 Plasmid map of pYPRC15-URA-UGPD-SmCYP_SmCPR. *pUC19*, pUC19 plasmid backbone; *YPRC-H1*, 500 bp homologous region upstream YPRC15 locus of *S. cerevisiae* genome; *YPRC-H2*, 500 bp homologous region downstream YPRC15 locus of *S. cerevisiae*; *eGPDp*, enhanced GPD promoter; *CYCt*, CYC terminator; *URA3*, URA3 marker; *SmCYP-SmCPR*, chimeric CYP-CPR gene, which is used as an example in this chapter and involved in oxygenation of miltiradiene.

pYPRC15_R with sequence "CATTTTGT*TTGTTTAT*GTG." CYP and CPR are fused in this design (linker's sequence is "GGCAGCA CCGGATCC"), and SmCYP and SmCPR are used as examples here (the sequence is shown in Sequence 2). Forward primer for SmCYP is SmCYP_F with sequence "ATAAACAA*ACAAAATG*GACTCTTTTCCATT ATTG"; reverse primer for SmCYP is SmCYP_R with sequence "GATCCGG*TGCTGCC*GGACTTGACGATTGG." Forward primer for SmCPR is SmCPR_F with sequence "GGCAGCA*CCGGATC* CGAACCATCCTCTAAAAA," and reverse primer for SmCPR is SmCPR_R with sequence "GATGGTGA*TGATGATG*CCAGACAT CTCTCAAGTA." pYPRC15-URA-UGPD, SmCYP, and SmCPR are amplified with corresponding primers and assembled according to procedure described in Sections 2.1.3–2.1.7.

4.1.2 Yeast Transformation

The constructed yeast expression vector is integrated into YPRC15 locus of *S. cerevisiae* BY4700 (MATa *ura3*Δ0) by using commercial kit 'S. c. EasyComp Transformation Kit' (Invitrogen) and following the manufacturer's instruction. Transformant is selected on YNB CSM-URA glucose agar plate (Section 4.1.3) – colonies usually appear after being incubated at 30°C for 2 days. A colony is inoculated into 10 mL of YNB CSM-URA glucose medium (Section 4.1.4) in a 50-mL Falcon tube, and incubated at 30°C/250 rpm for 2 days. Then 0.5 mL of the cell suspension is mixed with 0.5 mL of 80% glycerol solution (autoclaved) to make yeast glycerol stock, which can be stored at −80°C for up to 5 years.

SEQUENCE 2 Sequence of SmCYP_SmCPR

ATGGACTCTTTTCCATTATTGGCTGCCTTGTTTTTCATTGCTGCTACTATTACCTTCTTGT
CCTTCCGTAGAAGAAGAAATTTGCCACCAGGTCCATTTCCATATCCAATCGTTGGTAA
TATGTTGCAATTGGGTGCTAACCCACATCAAGTTTTTGCTAAGTTGTCTAAGAGATAC
GGTCCATTGATGTCCATTCATTTGGGTTCCTTGTACACCGTTATAGTCTCTTCACCAGA
AATGGCCAAAGAAATCTTGCATAGACATGGTCAAGTTTTCTCCGGTAGAACTATTGCT
CAAGCTGTTCATGCTTGTGATCACGATAAGATTTCTATGGGTTTTTTGCCAGTTGCCTC
TGAATGGAGAGATATGAGAAAGATCTGCAAAGAACAAATGTTCTCCAATCAATCCAT
GGAAGCTTCTCAAGGTTTGAGAAGACAAAAGTTGCAACAATTATTGGACCACGTCCA
AAAGTGTTCTGATTCTGGTAGAGCTGTTGATATTAGAGAAGCTGCTTTCATTACCACC
TTGAATTTGATGTCTGCTACCTTGTTTTCTTCACAAGCTACCGAATTTGATTCCAAGGC
TACCATGGAATTCAAAGAAATTATTGAAGGTGTTGCCACCATCGTTGGTGTTCCAAAT
TTTGCTGATTACTTCCCAATCTTAAGACCATTCGATCCACAAGGTGTTAAGAGAAGAG
CTGATGTTTTTTTCGGTAAGTTGTTGGCCAAGATCGAAGGTTATTTGAACGAAAGATT
GGAATCCAAGAGAGCTAATCCAAACGCTCCAAAGAAGGATGATTTCTTGGAAATCGT
TGTCGATATCATCCAAGCCAACGAATTCAAGTTGAAAACCCATCATTTCACCCACTTG
ATGTTGGATTTGTTTGTTGGTGGTTCTGATACCAACACCACTTCTATTGAATGGGCTAT
GTCTGAATTGGTTATGAACCCAGATAAGATGGCTAGATTGAAGGCTGAATTGAAATC
TGTTGCTGGTGACGAAAAGATCGTTGATGAATCTGCTATGCCAAAGTTGCCATACTTG
CAAGCTGTTATCAAAGAAGTCATGAGAATTCATCCACCTGGTCCTTTGTTGTTACCAA
GAAAAGCTGAATCCGATCAAGAAGTCAACGGTTACTTAATTCCAAAGGGTACTCAAA
TCTTGATTAACGCTTACGCCATTGGTAGAGATCCATCTATTTGGACTGATCCAGAAAC
TTTTGACCCAGAAAGATTCTTGGACAACAAGATCGATTTCAAGGGTCAAGACTACGA
ATTATTGCCATTTGGTTCAGGTAGAAGAGTTTGTCCAGGTATGCCATTGGCTACTAGA
ATATTGCATATGGCTACTGCTACTTTGGTTCACAATTTCGATTGGAAGTTGGAAGATG
ATTCTACTGCTGCTGCTGATCATGCTGGTGAATTATTTGGTGTTGCTGTTAGAAGAGC
AGTCCCATTGAGAATTATTCCAATCGTCAAGTCCGGCAGCACCGGATCCGAACCATC
CTCTAAAAAGTTGTCCCCATTGGATTTCATTACCGCCATTTTGAAGGGTGATATTGAA
GGTGTTGCTCCAAGAGGTGTTGCAGCTATGTTGATGGAAAACAGAGATTTGGCTATG
GTTTTGACTACCTCTGTTGCTGTTTTGATTGGTTGCGTTGTTGTTTTGGCTTGGAGAAG
AACTGCTGGTTCTGCTGGTAAAAAACAATTGCAACCACCAAAGTTGGTTGTTCCAAA
ACCAGCTGCTGAACCTGAAGAAGCTGAAGACGAAAAAACTAAGGTCAGTGTTTTCT
TCGGTACTCAAACTGGTACTGCTGAAGGTTTTGCTAAAGCTTTTGCCGAAGAAGCTA
AAGCTAGATATCCACAAGCTAAGTTCAAGGTTATCGATTTGGATGATTACGCTGCCG
ATGATGATGAATACGAAGAAAAGTTGAAGAAAGAATCCTTGGCCTTCTTCTTCTTGG
CTTCTTATGGTGATGGTGAACCTACTGATAATGCTGCTAGATTTTACAAGTGGTTCAC
CGAAGGTAAGGATAGAGAAGATTGGTTGAAGAACTTGCAATACGGTGTTTTTGGTTT
GGGTAACAGACAATACGAACACTTCAACAAGATTGCCATCGTTGTCGATGATTTGAT
TACTGAACAAGGTGGTAAGAAGTTGGTTCCAGTTGGTTTAGGTGATGATGATCAATG
CATCGAAGATGATTTTTCCGCTTGGAGAGAATTGGTTTGGCCAGAATTGGATAAGTT
GTTGAGAAATGAAGATGATGCTACTGTTGCTACTCCATATACCGCTGTTGTTTTACAA
TACAGAGTTGTCTTGCACGATCAAACTGATGGTTTGATCACAGAAAATGGTTCTCCAA
ATGGTCATGCTAACGGTAACACTATCTATGATGCTCAACATCCATGTAGAGCTAACG

Continued

SEQUENCE 2 Sequence of SmCYP_SmCPR—cont'd

TTGCTGTTAGAAGAGAATTGCATACTCCAGCTTCAGATAGATCTTGTACCCATTTGGA
ATTCGATACTTCAGGTACTGGTTTGGTTTACGAAACTGGTGATCATGTTGGTGTTTAC
TGCGAAAACTTGTTGGAAAATGTCGAAGAAGCCGAAAAGTTATTGAACTTGTCTCCA
CAAACCTACTTCTCCGTTCATACTGATAACGAAGATGGTACTCCATTGTCTGGTTCTT
CATTGCCACCACCATTTCCACCATGTACTTTGAGAACTGCTTTGACTAAGTACGCCGA
TTTGATTTCTATGCCAAAGAAGTCTGTTTTGGTTGCTTTGGCTGAATACGCCTCTAATC
AATCAGAAGCTGATAGATTGAGATACTTGGCTTCACCAGATGGTAAAGAAGAATACG
CCCAATATATCGTTGCCTCCCAAAGATCATTATTGGAAGTTATGGCTGAATTCCCATC
TGCTAAACCACCATTGGGTGTTTTTTTTGCTGCTATTGCTCCTAGATTGCAACCTAGAT
TCTACTCCATTTCTTCCTCCCCAAAAATTGCTCCAACTAGAGTTCATGTTACCTGTGCT
TTGGTTTATGATAAGACTCCAACTGGTAGAATCCATAAGGGTATTTGTTCTACCTGGA
TTAAGAACGCTGTTCCATTGGAAGAATCTTCAGATTGCTCTTGGGCTCCAATTTTCATC
AGAAACTCTAACTTTAAGTTGCCAGCCGATCCAAAGGTTCCAATTATCATGGTTGGTC
CAGGTACAGGTTTAGCTCCTTTTAGAGGTTTCTTACAAGAAAGATTGGCCTTGAAAGA
ATCTGGTGCTGAATTGGGTCCAGCTATTTTGTTTTTTGGTTGTAGAAACAGAAAGATGG
ACTTCATATACGAAGATGAATTGAACTCCTTCGTTAAGGTTGGTGCCATTTCTGAATTG
ATCGTTGCTTTTTCTAGAGAAGGTCCAGCCAAAGAATACGTTCAACATAAGATGTCTC
AAAGAGCCTCCGATATTTGGAAGATGATATCTGATGGTGGTTACATGTACGTTTGTGG
TGATGCTAAAGGTATGGCTAGAGATGTTCATAGAACCTTGCATACCATTGCTCAAGAA
CAAGGTTCTTTGTCATCTTCTGAAGCAGAAGGTATGGTCAAAAACTTGCAAACTACTG
GTAGATACTTGAGAGATGTCTGGCATCATCATCACCATCACTAA

4.1.3 YNB CSM-URA Glucose Agar Plate

Seven-point 5 g of agar is mixed with 350 mL of deionized water and autoclaved. When it is cooled down to 60°C, 50 mL of 10 × YNB solution (with ammonium sulfate, filtration sterilized), 50 mL of 10 × CSM-URA solution (complete synthetic medium with uracil dropped off, autoclaved), and 50 mL of 200 g/L glucose (autoclaved) are added. Twenty milliliters of the mixture are poured into a 90-mm petri dish to make an YNB CSM-URA glucose agar plate.

4.1.4 YNB CSM-URA Glucose Medium

Fifty milliliters of 10 × YNB solution (with ammonium sulfate, filtration sterilized), 50 mL of 10 × CSM-URA solution (complete synthetic medium with uracil dropped off, autoclaved), 50 mL of 200 g/L glucose (autoclaved), and 350 mL of deionized water (autoclaved) are mixed to make 500 mL of YNB CSM-URA glucose medium.

4.2 Characterization of the *S. cerevisiae*

Activity of the CYP expressed in *S. cerevisiae* can be quantified by feeding its substrate (ISM) into its culture and monitoring substrate oxygenation.

4.2.1 Preparation of Isoprenoid-DMSO Stock Solution

C20 isoprenoid scaffold is highly soluble in DMSO, a solvent that does not affect yeast growth when its concentration is less than 1% (v/v) in yeast culture. Two milligrams of the HPLC-purified isoprenoid (Section 3.2.5) are dissolved in 0.4 mL of DMSO (5 g/L of isoprenoid), and this solution is sterilized by using 13 mm 0.2 μm Nylon filter.

4.2.2 Preparation of Seed Culture

Small quantity of yeast glycerol stock is inoculated into 1 mL of YNB CSM-URA glucose medium – held in 14 mL Falcon tube – by using disposable 1 μL inoculation loop. The tube is incubated at 30°C/250 rpm for 2 days.

4.2.3 Feeding Isoprenoid into Yeast Culture

Fifteen microliters of fully grown yeast culture is inoculated into 1.5 mL of YPD medium in a sterile VWR 16 × 100 mm (diameter and height) round-bottom culture tube and incubated at 30°C/250 rpm. When cell density reached 1 (OD600), 15 μL of the isoprenoid-DMSO stock solution is added. The incubation continued for 96 h, and 100 μL of cell suspension is taken every 24 h for GCMS analysis, which is carried out as described in Sections 2.2.7–2.2.8.

4.3 Coculture of the *S. cerevisiae* with an Isoprenoid-Overproducing *E. coli*

4.3.1 Preparation of Yeast Preculture

The *S. cerevisiae* strain constructed in Section 4.1.2 and the best *E. coli* strain obtained in Section 2.2.6 are cocultured in the same bioreactor. To ensure their coexistence, they are cultured in a medium which contains xylose as the sole carbon source. The two microbes thus have a mutualistic relationship in this case: *S. cerevisiae* cannot utilize xylose and relies on *E. coli* to access carbon; *E. coli* naturally produces acetate – a by-product inhibitory to its own growth – and depends on *S. cerevisiae* to remove it. To ensure no acetate accumulation in bioreactor, an excess of yeast relative to *E. coli* needs to be inoculated and the needed preculture is prepared by addition of 1 mL of fully grown seed culture into 50 mL of YPD, followed by incubation at 30°C/250 rpm for 24 h.

4.3.2 Bioreactor Run

Bioreactor used is the same to that described in Section 3.1, and it is prepared according to protocol in Section 3.2.1. The bioreactor is initiated and operated as described in Section 3.2.2 except that pellet of 50 mL of fully grown yeast preculture is inoculated together with the *E. coli* seed culture – the yeast pellet is resuspended in the *E. coli* seed culture prior to inoculation. Feeding strategy applied here is the same to that in Section 3.2.3. Sample is taken every 24 h for cell density, GCMS, and HPLC analysis. GCMS samples are processed as described in Sections 2.2.7–2.2.8, and HPLC samples are processed according to Section 3.2.4. For cell density analysis, 500 μL of coculture are loaded onto 1 mL of 45% sucrose solution in a 14-mL Falcon tube, and the tube is centrifuged at $2100 \times g$ for 2 min. After centrifugation, all yeast cells are pelleted and most *E. coli* cells remain in supernatant. Then cell density of each species can be quantified by measuring absorbance at 600 nm (OD600).

5. DISCUSSION

Protocols provided in this chapter need to be revised for producing other types of isoprenoids. Plasmids for expressing IsoSs in this chapter all contain GGPP synthase (Fig. 4), which needs to be replaced if target isoprenoids are derived from FPP, GPP, DMAPP, or IPP. Isoprenoids with larger backbones (C30, C40, etc.) generally cannot diffuse through cell membranes, thus their biosynthetic pathway cannot be distributed between two cells. The small isoprenoids (C5 and C10) are very volatile, so they should be recovered from off-gas exit of the bioreactor or trapped in the bioreactor by using a nontoxic organic solvent such as dodecane. C15 isoprenoids have similar properties to the C20 ones; hence, the same bioreactor configuration and operating conditions can be applied to this type of isoprenoids.

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